Title	Novel Cell-Based Therapies for the Treatment of Diabetic Ulceration
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Publication Date	2012-02-28
Item record	http://hdl.handle.net/10379/2731

Novel Cell-Based Therapies for the Treatment of

Diabetic Ulceration

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Submission: December 2011

Table of Contents

Summary

The focus of this research is on developing novel cell based therapies for the treatment of non-healing diabetic foot ulceration. The thesis begins with a concise review of the diabetic foot ulcers. The burden associated with non-healing diabetic foot ulceration in humans is presented. The biology of the diabetic ulcer is reviewed and the scientific rationale for pursuing the development of a stem and progenitor cell-based therapy is emphasised. Cell transplantation using biomaterials is reviewed.

The first cell type that is investigated is the early endothelial progenitor cell or circulating angiogenic cell. A cell-scaffold treatment was developed using collagen. A preclinical model of diabetic wound healing was validated. The model is the alloxan induced diabetic rabbit ear ulcer model. Subsequently autologous circulating angiogenic cells exposed to the matricellular protein osteopontin were applied to a full thickness cutaneous ulcer. The cells were delivered via a collagen scaffold and the percentage wound closure was assessed after one week. Circulating angiogenic cells exposed to osteopontin and seeded on a collagen scaffold displayed significantly increased percentage wound closure as compared to other groups. Stereological analysis of wounds demonstrated a superior vascular network in wounds treated with circulating angiogenic cells exposed to osteopontin. The secretome of human circulating angiogenic cells was assessed with diabetic CACs demonstrating less angiogenesis in vitro.

Topical treatment of ulcers with allogeneic non-diabetic mesenchymal stem cells was assessed in the same preclinical model. A dose escalation protocol was carried out. Wounds treated with 1,000,000 MSCs seeded in a collagen scaffold augmented wound healing as compared to untreated wounds. Stereological analysis demonstrated a superior neovasculature in wounds treated with 1,000,000 MSCs

The outcome of this research is the demonstration of the therapeutic efficacy of topically applied circulating angiogenic cells and mesenchymal cells in diabetic cutaneous ulceration.

Acknowledgements

I would like to dedicate this thesis to Dawn who showed endless support and love and without whom I could not have completed the project.

I would like to also dedicate it to my Mum and Dad for their ongoing help and support

To my supervisors I would like to extend my sincere thanks for advice and support and providing me with the opportunity to undertake this research.

To Molecular Medicine Ireland for providing me with the opportunity and funding to pursue a structured clinical scientist trainee PhD program

To all who helped me with this research, all the staff in REMEDI and NFB for all their help and support, especially Mangesh and Erin, and all the staff in the diabetes centre, the patients and healthy' volunteers.

Abbreviations

AGE	Advanced Glycation Endproducts
ATMP	Advanced Therapy Medicinal Product
ATSC	Adispose Tissue-derived stromal cells
BM	Bone Marrow
CAC	Circulating Angiogenic Cell
CD	Cluster Differentiation
CM	Conditioned Media
CM-DiI	Chloromethylbenzamido-1,1-dilinoleyl-3,3,3',3' tetramethylindocarbocyanine perchlorate
d	Blood Vessel Diameter
14S,21R-diHDHA	4S,21R-dihydroxydocosahexaenoic acid
DiI-Ac-LDL	1,1-dilinoleyl-3,3,3',3' tetramethylindocarbocyanine perchlorate – acetylated-low-density lipoprotein
DFU	Diabetic Foot Ulcer
CXCL4	Chemokine (C-X-C Motif) Ligand 4
CXCL16	Chemokine (C-X-C-Motif) Ligand 16
CXCR4	Chemokine (C-X-C-Motif) Receptor 4
EDTA	Ethylenediaminetetraacetic Acid
EPC	Endothelial Progenitor Cell
EBM	Endothelial Based Media
EMA	European Medicines Agency
EU	European Union
FDA	Food and Drug Administration
FITC- UAE Lectin	Fluorecein isothiocyanate-conjugated Ulex Agglutinin Europaeus lectin (FITC- UAE-lectin).
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
GMP	Good Manufacturing Process
GT	Granulation Tissue
GFR	Glomerular Filtration Rate
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HANKS	'Hanks' Balanced Salt solution

HbA1c	Glycated Haemoglobin
HBO	Hyperbaric Oxygen Therapy
H+E	Haematoxylin + Eosin
HIPE	Hospital Inpatient Enquiry
HUVEC	Human Umbilical Vein Endothelial Cell
HRP	Horseradish peroxidase
Il-8	Interleukin-8
L_v	Length Density
MHC	Major Histocompatibility Complex
MRI	Magnetic Resonance Imaging
MSC	Mesenchymal Stem Cell
MMP-9	Matrix Metalloproteinase-9
OPN	Osteopontin
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PKH-26	Red Fluorescent Cell Linker Kit for Cell Labeling
R_{diff}	Radial Diffusion Distance
RAGE	Receptor for advanced glycation end products connective
PARP	Poly(adenosine diphosphote)-ribose polymerase
PEDF	Pigment Epithelium-derived Factor
REMEDI	Regenerative Medicine Institute
SD	Standard Deviation
SDF	Stromal-Derived Factor
S_v	Surface Density
T_cPO_2	Transcutaneous oxygen tension
TRITC	Tetramethyl Rhodamine Isothiocyanate
TIMP-1	Tissue Inhibitor of Matrix Metalloproteinase-1
TSP-1	Thrombospondin-1
VEGFR2	Vascular Endothelial Growth Factor Receptor-2
V_v	Volume Fraction

Aonghus O'Loughlin performed this work by himself, and it was not done by other people. Advice and supervision was obtained.

Chapter 1.

Basic Concepts to Novel Therapies: A Comprehensive Review of the Diabetic Foot

Diabetes Mellitus is a global epidemic. Peripheral neuropathy and peripheral vascular disease are complications of diabetes mellitus and the primary causative factors for foot ulceration. Foot ulceration is the leading cause of hospitalisation in people with diabetes mellitus. The burden of foot ulceration on healthcare systems and individual patients is immense. Despite conventional treatment, there persists a high incidence of amputation. A multidisciplinary approach is required to prevent ulcers. This review describes the aetiology and risk factors for diabetic foot ulceration and a system for evaluating the diabetic foot. The assessment of neuropathy and the grading of foot ulcers are critically examined. This is important to allow for standardisation in clinical trials. The management of diabetic foot syndrome is reviewed. The treatments to ensure vascular supply to the lower limb and control of infection are described. Novel therapies, which are becoming available to treat non-healing, 'no-option' diabetic ulcers are discussed.

Introduction

There has been a substantial increase in the prevalence of diabetes mellitus worldwide and it is predicted that this will continue resulting in a substantial financial burden. Diabetic foot ulcers affect 12 to 25 percent of persons with diabetes mellitus throughout their lives.[1] Lower limb disease is the most common source of complications and hospitalisation in the diabetic population.[2] The estimated cost of treating a diabetic foot ulcer over 2 years is $28,000.[3] In Ireland, the inpatient cost of treating a diabetic ulcer over one year was estimated at €23,500 per patient.[4] In addition, leg ulceration has a significant impact on the quality of life of the patient. The parameters of pain, social isolation, physical morbidity, restrictions in work capacity and psychological well-being are negatively affected by leg ulceration.[5] Specifically with diabetic foot ulcers, health related quality of life scores improved significantly in social functioning and mental health in parallel with healing of ulcers, and deteriorated in the social functioning subscale in parallel with non-healing of ulcers.[6] In addition with diabetic foot ulcers, there is an association between severity i.e. biochemical signs of inflammation c-reactive protein >10 mg/l, ankle-brachial index <0.9, ulcer size >5 cm^2, and health related quality of life on physical functioning.[7] There is also an increased incidence of depression in patients with diabetic foot ulceration, with diabetic peripheral neuropathy a significant risk factor due to pain and unsteadiness.[8,9]

Neuropathy and peripheral vascular disease are major factors in the pathogenesis of diabetic foot ulcers. The combination of these and an increased risk of infection predispose to foot ulcers. However there are a large number of factors leading to the occurrence of foot complications. These include uncontrolled cardiovascular risk factors i.e. diabetes, blood pressure, lipids and obesity. Smoking, increasing age, longer duration of diabetes and improper prevention strategies also play key roles. Recent evidence has demonstrated an association between chronic kidney disease and lower extremity amputation in people with diabetes.

Major lower limb amputations in patients with diabetes arise from preceding ulcers in 85% of cases.[10] The annual incidence of foot ulceration is greater than 2% percent among all patients with diabetes and between 5.0% and 7.6% among patients with peripheral neuropathy.[11-13] These complications are largely preventable with the correct medical care as will be discussed in this article.

Aetiology of Diabetic Foot ulcers

The aetiology of diabetic foot ulcers is multi-factorial. Diabetic neuropathy is central and present in the majority of patients. Distal symmetrical polyneuropathy has been reported as the primary cause of plantar ulceration. [2,14] Nerve damage involves sensory, motor and autonomic nerves and subsequently the patients' ability to perceive pain, pressure, touch and temperature is altered. Altered proprioception makes the patient unable to determine the position of the foot. Motor neuropathy affects the small muscles of the foot and causes weakness, atrophy and deformity. The deformities include clawing of the toes, prominent metatarsal heads and limited joint mobility. Autonomic neuropathy may reduce sweating and increase the temperature of the foot, predisposing to infection.[15] The reduction in sweating and increased temperature predispose to cracking of the skin and consequent ulceration.

The most common mechanism of injury results from repetitive unperceived pressure on bony prominences and this is potentiated by pre-existing deformity.[16,17] Poorly fitting footwear, small foreign bodies in footwear and puncture wounds produce pressure necrosis and lead to ulceration.[18,19] Structural deformities are common sites of abnormally high pressure and repetitive pressure at these sites can result in tissue breakdown.[20] There is evidence that ulcers develop at sites of maximal pressure.[21-23] A combination of lack of sensation, limited joint mobility, autonomic dysfunction resulting in dry skin and high pressure leads to callus formation.[24] Callosities develop at high pressure sites and in the absence of protective sensation continued activity can cause the callosities to thicken, haemorrhage underneath and eventually ulcerate. Painless callosity formation suggests neuropathy. The relative risk of ulcer development at an area

of high pressure (i.e. the metatarsal heads as compared with the mid-foot) is 4.7 and that of an ulcer developing at a site of callus is 11.0.[25]

Charcot`s neurarthropathy is the result of bony dislocation and collapse of the arch.[26] Autonomic dysfunction is implicated with abnormal perfusion to foot bones. The `rocker-bottom` deformity is prone to increased pressure and ulceration.[27]

Foot ulcers in patients with diabetes mellitus often have mixed ischaemic and neuropathic components. Arterial insufficiency occurs in the diabetic lower limb. Wound healing depends on an adequate blood supply and ischemia impairs healing by reducing the supply of oxygen and nutrients and soluble mediators that are involved in the repair process.[28] Arterial insufficiency is a pathogenic factor in up to 60% of diabetic patients with non-healing ulcers and in 46 percent of those undergoing amputation.[29] Atherosclerotic lesions typically affect distal peroneal and tibial arteries.[18] Defects in the microcirculation of the diabetic foot have been implicated in the pathogenesis of diabetic ulcers. Hyperglycaemia induced nerve dysfunction leads to dysregulation of nerve microvasculature and consequent neuropathy. The sustained viability of the skin in people with diabetes relies on a functioning microcirculation. There exists several proposed mechanisms altered skin blood and nerve dysfunction and are reviewed in a recent review.[30] The skin microcirculation may be assessed by several techniques e.g. transcutaneous oxygen tension measurement, photoplethysmosgraphy, laser doppler flowmetry, laser Doppler imaging and orthogonal polarization spectral imaging.[30]

Patients with diabetes mellitus are more prone to infections. Infection is usually a consequence rather than a cause of ulceration. Infection prolongs ulceration by allowing the entry and multiplication of microorganisms.[31] The presence of a foot infection is broadly accepted as the presence of systemic signs of infection or purulent secretions, or two or more local symptoms (redness, warmth, induration, pain or tenderness).[31,32] Osteomyelits may affect up to two thirds of patient with diabetic ulcers and must be considered.[33] In diabetic foot ulceration osteomyelitis may occur without pain. Sinus formation is present with or without systemic

signs. It is important to note that local signs of infection can be diminished due to peripheral arterial disease and neuropathy.

Risk Factors for ulceration

Boyko et al. evaluated readily available clinical information in the prediction of diabetic ulcers. Significant predictors included elevated HbA1c, impaired vision, prior foot ulcers, prior amputation, monofilament insensitivity, tinea pedis and onychomycosis.[2]

The American Diabetes Association position statement on Foot care identifies people at higher risk of foot ulceration and amputations as those who have diabetes for over 10 years, male sex, poor glucose control and cardiovascular, retinal or renal complications. The following foot conditions are associated with an increased risk of amputation: proprioceptive loss, altered biomechanics, evidence of increased pressure (erythema, haemorrhage under a callous), bony deformity, peripheral vascular disease (decreased or absent pedal pulses), a history of ulcers or amputation and severe nail pathology.[34] There is an increased risk of amputation in patients with a reduced glomerular filtration rate.[35]

Table1 summarises data from a review of 95 cases of diabetes-related lower extremity amputation identified from a hospital inpatient enquiry (HIPE) database search. HIPE is a database used by Irish hospitals to collect information on discharges of patients treated in the inpatient hospital setting.

Table 1. Demographics and risk factors of patients undergoing diabetes-related lower extremity amputation between 2004 and 2008.

Gender	Male 74%; Female 26%
Age (years)[a]	72 +/- 10.6
Type of Diabetes	T1 DM 12.7%;T2 DM 87.3%
Duration of Diabetes (years)[a]	15.1 +/- 13.1
HbA1c[a]	7.88 % +/- 1.87
Peripheral Vascular Disease[b]	80 %
Neuro-ischaemic ulcers	45 %
Neuropathic ulcers[c]	12 %
Smoking (current or past smoker)	71%
GFR < 60 mL/min/1.73 m^{2}[d]	37.5%
Previous Amputation	38%

[a]For Age, Duration of diabetes and HbA1c, data are presented as mean values +/- Standard Deviation
[b]Peripheral Vascular Disease is defined as, absent peripheral pulses or reduced ankle Brachial Index or reduced toe pressures and/or MRI, CT, angiography evidence of peripheral vascular stenosis.
[c]Available data from patients who had urea and electrolytes measured at routine diabetes outpatients or general practice clinics on two occasions. GFR: Glomerular filtration rate calculated by Modified Diet in Renal Disease equation
[d]Neuropathic ulcers identified by evidence of documentation in the medical notes

How to prevent amputation

The following sections of the review highlight the best strategies to prevent diabetes-related lower extremity amputation using an evidence-based approach. The basic concepts of routine examination and evaluation of the diabetic foot with identification of risk factors and neuropathy are discussed. The benefits of education, podiatry, and orthotics are presented with reference to key research articles. Further evaluation and treatment of the insensate foot, diminished vascular supply and infection are reviewed. The role of wound care and negative pressure therapy is discussed. The review concludes with a discussion of novel therapies which are providing therapeutic alternatives for non-healing diabetic foot ulcers that are not responding to conventional treatment. A key message is

the requirement of a multidisciplinary approach to management, with the use of treatment algorithms and care pathways.

Evaluation of the Diabetic Foot

Evidence of neuropathy or peripheral vascular disease should be sought in the history. Burning, numbness, tingling, fatigue, cramping or aching, location of the symptoms, nocturnal symptoms and what relieves the symptoms should be sought.

A comprehensive foot examination is necessary in people with diabetes mellitus. People with type 2 diabetes mellitus may have evidence of neuropathy at the time of diagnosis. Visual inspection of the feet is required for erythema, callus formation, deformity, skin integrity and fungal infections of skin and nails. Gait, balance and foot wear must be assessed. Examination of the vascular status of the foot includes palpation of pulses, and if not present the use of a Doppler ultrasound is required.

The neuropathic foot is warm and well perfused with palpable pulses, sweating is diminished and the skin may be dry and prone to fissuring. The neuro-ischaemic foot is cool, pulseless with thin shiny skin without hair and atrophy of the subcutaneous muscle.[36]

Measurement of cutaneous pressure perception with the use of Semmes-Weinstein monofilament is widely used and is a rapid, easy clinical test. It is a validated screening test for neuropathy and ulcer potential because of simplicity, sensitivity and low cost.[37-41] The loss of pressure sensation at four sites, as detected by the buckling of a 10-g monofilament is highly predictive of subsequent ulceration. The four sites include the 1st, 3rd and 5th metatarsal heads and plantar surface of distal hallux.[42]

Vibration testing with a 128-Hz tuning fork applied at a bony prominence is a useful test for peripheral neuropathy with a sensitivity and specificity of 53 and 99 percent respectively.[43] The ankle jerk and patellar reflexes are examined.

Quantification and grading systems of neuropathy and ulcers

There are quantitative grading systems for ulcers and neuropathy in addition to more technical modalities available for pressure testing which are used more often in specialised centres. The McGill pain questionnaire and neuropathy symptom profile (NSP) or neuropathy change score (NCS) may be used to grade symptoms of neuropathy and are commonly used in clinical trials.[44-47] Improvement in symptoms does not correlate with improvement in nerve function.[48,49] The Michigan Neuropathy Screening instrument (MNSI) is used for staging and diagnosing peripheral neuropathy. It is a questionnaire and clinical examination and if there is an abnormal score the patient is referred for quantitative sensory testing (semmes-weinstein monofilaments, two-point discrimination, vibration perception, thermal and cooling thresholds, computer assisted sensory examination) and electrophysiological studies.[50] The neuropathy disability score (NDS) is used for assessment of neurological deficits secondary to neuropathy.[51] Both may be used in studies on diabetic neuropathy.[50,52]

Electrophysiological studies examining motor and sensory nerve conduction velocities are an important efficacy parameter and are objective, sensitive and reproducible. They are not a specific investigation of diabetic neuropathy. They are frequently used as surrogate endpoints in clinical trials. Composite of clinical, quantitative, sensory and electrophysiological measure scores include MNSI and NISLL (neuropathy impairment score of the lower limbs).[53]

There is no standardised scoring system for ulcers. The University of Texas system appears to be a good predictor of outcome and includes the variables of size and depth of ulcer and the presence or absence of infection or ischaemia.[54,55] The Wagner grading system is also used for foot ulcers but does not incorporate the presence of co-existent infection.[56] A recent consensus statement details hard endpoints for use in clinical trials of wound healing treatments.[57] The assessment of the vascular supply of the foot is dealt with below. The pathophysiology of diabetic neuropathy is complex and not fully understood and is beyond the scope of this review.

Management of the Diabetic Foot and ulcers

Regular Examination

Each patient with diabetes mellitus requires a comprehensive foot examination annually to identify risk factors for neuropathy and any evidence of neuropathy or ulceration. There may be an absence of symptoms in patients with diabetic foot disease and screening with foot examination is essential. Boulton *et al*. describes risk assessment and treatment recommendations for the management of the diabetic foot. The risk assessment used is based on loss of peripheral sensation and peripheral vascular disease with the highest risk based on history of ulcer or amputation. The frequency of clinical review depends on a patient's risk score. The indications for implementation of prescriptive and accommodative footwear depend on risk and should be considered in patients with loss of protective sensation, deformity and the presence of peripheral arterial disease.[42]

Multidisciplinary Care

A limb salvage team with a multidisciplinary approach is required with diabetologists, diabetes specialist nurses, podiatrists, vascular surgeons, orthopaedic surgeons, rehabilitation specialists, physiotherapists, occupational therapists and infectious disease specialists. It has been reported that higher levels of feedback coordination between specialities resulted in lower amputation rates.[58] Foot lesions are more likely to lead to amputation in the absence of a multidisciplinary team.[59,60] These teams allow for intensive treatment and rapid access to orthopaedic and vascular surgery for revascularisation and control of infection. They also aid in rehabilitation and ultimately positively affect quality of life.

Education

Each patient with diabetes mellitus should be educated on the complication of diabetic neuropathy and ulceration. The patient with diabetes mellitus should understand the implications of loss of protective sensation and the benefit in foot

monitoring on a daily basis. The patient is advised on the appearance of erythema, callus, infections and nail problems. This information should be impressed on the family members and partners of the patient on agreement with the patient. The importance of avoiding walking barefoot and potential trauma is impressed on the patient.

In order to reduce the vascular compromise associated with diabetes in the lower limb, the patient should refrain from cigarette smoking, exercise regularly, maintain ideal weight and maintain target levels for lipids, glycaemic control and blood pressure. Improvements in HbA1c reduces the incidence of neuropathy as shown in the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS).[61-63]

Teaching patients to monitor their risk factors retards the progression of vascular complications in high risk patients with type 2 diabetes. A Cochrane review also examined education of health professionals for preventing diabetic foot ulceration and this concluded added benefit.[64] A systematic review of screening and education for high risk individuals reported mixed results and the need for more randomised controlled trials.[65]

Footwear

Appropriate footwear is essential. The footwear should be of adequate width and depth. Custom-made foot orthoses reduce plantar callus thickness and the incidence of ulcer relapse and should be used.[66] These custom made footwear should be considered in patient's with evidence of deformity with or without loss of peripheral sensation.[42] The use of custom moulded shoes is required in people with severe deformity or partial amputation.[67] There has also been conflicting evidence on the use of therapeutic shoes and orthotic devices and total contact casts. A systematic review from Spencer in 2000 concluded the need to measure a range of pressure relieving devices in prevention and treatment of diabetic foot ulcers as there is limited evidence base in this area.[68] Bus *et al*. have published guidelines on footwear and offloading for the diabetic foot which is based on

conclusions from the systematic review on available evidence and on consensus agreement within the International Working Group on the Diabetic Foot.[69]

Debridement

The recognition and use of debridement in treating ulcers and calluses is required. It should be done with caution in the ischaemic foot so as not to damage viable tissue. Debridement improves ulcer healing time. The procedure should only be undertaken by a health professional who is skilled and has received sufficient training in the technique. Sharp debridement has been the most thoroughly studied and regular sharp debridement every week is associated with more rapid healing of ulcers than less frequent debridement.[70] A systematic review on debridement of diabetic foot ulcers reported that hydrogel increases healing rate of ulcers compared with gauze dressing or standard care. Larval therapy significantly reduced wound area, but more research is needed to evaluate the range of debridement methods available.[71] Hydrogel is not appropriate for moderately or heavily exuding wounds or ischaemic wounds. The percentage change in foot ulcer area at 4 weeks is a robust predictor of healing at 12 weeks and is a good indicator of success of therapy and need for a different modality of treatment.[72] Sharp debridement followed by intermittent foot compression by a pneumatic pump resulted in higher healing rates than sharp debridement alone.[73] This approach aids in the control of local oedema.

Podiatry

A podiatry clinic provides the patient with education and regular debridement. Regular podiatric care and secondary prevention measures by a podiatrist may reduce recurrence of foot ulcers. Access to podiatric services reduces the number and size of foot calluses and improves self-care. Plank *et al.* and Ronnemaa *et al.* report in randomised controlled trials that podiatry input reduces amputation rates.[74,75]

Removal of pressure

The removal of mechanical pressure from a neuropathic foot ulcer is central to the healing of the ulcer and its role is well established.[76] Resting the foot is the ideal way of removing pressure from the wound, however this is difficult if the patient is working or has to mobilise for any reason. The footwear mentioned above is useful in removing pressure as well as felted foam dressing. The immobility associated with pressure relieving treatments may negatively impact on coronary heart disease due to lack of exercise.

The use of a non-removable, total contact cast is superior standard therapy in removing pressure. It is associated with more rapid healing rates.[77] It also allows better adherence to weight relieving strategies as the patient may not remove the cast and walks less frequently. They are normally in place for 12 weeks and are kept under regular review in that time. In most circumstances, the cast is refitted every week, with inspection and debridement usually occurring every one to two weeks. A systematic review by Bus et al. reports a lack of standardisation in terminology, prescription, manufacture, and material properties of footwear and offloading devices.[76] The use of these casts should be considered for unilateral plantar ulcers to optimise rate of healing. A randomised controlled trial reported a reduction in healing time to a mean of approximately 6 weeks in patients with unilateral diabetic foot ulcers.[78] They are more commonly used in the treatment of Charcot's foot and remove pressure and reduce temperature. Ongoing infection and ischaemia are contraindications to permanent casts.

Dressings

There is much research and development in the area of dressings that promote wound healing. The use of moist dressings on clean granulating wounds improves the wound environment.[32] These dressings provide protection against further infection and maintain moisture balance, wound pH, absorb fibrinous fluids and reduce local pain. The properties of the dressing should match the characteristics of the ulcer. The choice of dressing is further guided by patient requirements and costs.[79] Moist dressings promote healing, but are used in less than 50% chronic wounds. The use of moist wound dressings allows for faster

healing, improved tissue quality and less scarring but normal saline moist to dry dressings may cause non-selective tissue destruction and maceration.[80-84] Several trials have looked at cellulose and protease modulating dressings including Hyaluron dressings but have not been assessed in well designed trials.[85] A systematic review investigating silver based dressings found insufficient evidence to establish whether silver-containing dressings or topical agents promote wound healing or prevent wound infection.[86]

The use of negative pressure wound therapy by increases the rate of ulcer healing. Vacuum assisted closure (VAC) devices appear to be as safe as and more efficacious than advanced moist wound treatments such as hydrogels and alginates for the treatment of diabetic foot ulcers.[87-89] It can augment angiogenesis, granulation tissue formation and lead to cell proliferation. It is a safe therapy, and is available as a portable device allowing use in the outpatient setting. This may reduce hospital stay, and reduce wound dressings. The device is worn with the dressing changed every 48 hours, and the therapy should not be removed for over two hours. Negative pressure wound therapy delivered by the VAC therapy system seems to be a safe and effective treatment for complex diabetic foot wounds, and could lead to a higher proportion of healed wounds, faster healing rates, and potentially fewer re-amputations than standard care.[90] Its' use is contraindicated in untreated osteomyelitis.

Infection

While diabetic neuropathy is the major cause of foot ulceration, infection is the final common denominator that leads most people to amputation.[91] There is much variability in treatment approaches to infected diabetic foot ulcers and this emphasises the need for evidence-based guidelines. The degree of infection may be classified as grade, 1 (none), 2 (superficial), 3 (extensive erythema/depth) and 4 the presence of systemic manifestations.[92] There is also benefit in culturing the micro-organism to allow targeted antibiotic treatment. A superficial swab is not effective as a skin wound harbours many micro-organisms. A deep tissue specimen is more useful as is aspirated purulent material. There is variability in culture results between patient populations. Aerobic gram positive organisms

predominate, particularly *Staphyloccus Aureus*. The other common aerobe includes *streptococcus*. Polymicrobial isolates are common, particularly in chronic diabetic foot ulcers and include both aerobic and anaerobic species. The choice of antibiotic depends on cultured pathogens.

Commonly used antibiotics include clindamycin, ciprofloxacin, co-amoxyclav until cultures are available and if deeper infection is considered polymicrobial cover for gram positive cocci, gram negative bacilli and anaerobes should be continued. Soft tissue infections usually require 2 weeks of therapy and if the infection does not respond to antibiotics, therapy should be changed depending on culture results. A protocol for treating diabetic foot infections has been proposed but there is no internationally agreed protocol.[93] A systematic review concluded that the evidence is too weak to recommend any particular antimicrobial agent for diabetic ulceration.[94]

Osteomyelitis may occur in two thirds of diabetic patients with foot ulcers.[31] Histological and microbiological diagnosis from aseptic bone culture is the gold standard. MRI scanning is regarded as the test of choice for suspected osteomyelitis with sensitivity and specificity in diabetic patients of 90 % or greater.[95] Plain radiographs are neither sensitive nor specific but are a useful non-expensive test especially if able to compare with previous films. Isotope bone scans are sensitive but lack specificity. The ability to probe to bone in an ulcer with a stainless steel probe has a positive predictive value of 89% and a negative predictive value of 56% for osteomyelitis in one study, and another recent study revealed a positive predictive value of 57% with a negative predictive value of 98%.[96,97] An ulcer greater than 2 cm in diameter, an erythrocyte sedimentation rate greater than 70 mm/h, may also be helpful in determining the likelihood of osteomyelitis.[98] *Staphylococcus Aureus* is the most common pathogen typically arising from direct inoculation of bone.

Treatment of osteomyelitis requires intravenous antibiotics depending on bone culture. The optimal duration of antibiotics either intravenously or orally is not known. Osteomyelitis may require more than 6 weeks therapy and surgical

debridement of bone. Early referrals to orthopaedic and an infectious disease specialist are recommended.[31]

Vascular supply

All patients with tissue loss and arterial disease should be considered for arterial reconstruction. In patients with extensive tissue loss or gangrene of the foot, restoration of pulsatile blood flow to the foot is required for healing.[29] The patient with peripheral vascular disease as evidenced by diminished pulses should have non-invasive testing performed initially. The non-invasive tests include ankle-brachial index, digital toe pressures, waveform, colour duplex analysis and transcutaneous oxygen. Current guidelines for management of the diabetic foot include assessment for peripheral vascular disease. The presence or absence of peripheral vascular can determines which risk category the patient fits into.[42] Transcutaneous oxygen tension ($TcPO_2$) assessment is a simple, noninvasive and reliable technique for the investigation of arterial occlusive disease in the legs. It measures the amount of the oxygen delivered to the skin, allowing objective quantification of the degree of limb ischaemia. It is reported that a $TcPO_2$ level greater than 30 mmHg proved a strong predictor of spontaneous wound healing in patients with diabetic foot ulceration.[99]

Referral to vascular surgery allows for assessment and re-vascularistion procedures. The other investigations include MR angiography and arteriography. The effective revascularisation procedures include angioplasty (balloon and laser), stenting, atherectomy and bypass grafts to foot vessels. If vascular reconstruction is not possible gangrenous toes may be allowed to auto-amputate.

Results of a local audit carried out on 95 inpatients undergoing treatment for non-healing diabetic foot ulcers which required amputation from 2004 to 2008 demonstrated the performance of 54 balloon angioplasties and 9 endovascular stent placements. There was history of 24 distal bypass operations, performed either before or during the 4 year analysis period.

Increased frequency of distal bypass is associated with reduced risk of amputation, however recent research highlights that people with diabetes undergoing leg bypass surgery for critical limb ischaemia have a 55 % increased risk for major amputation or death compared to those without diabetes.[100,101] The BASIL trial investigated the benefit of angioplasty or leg bypass surgery for severe limb ischaemia due to infra-inguinal atherosclerotic disease. The results revealed that either method could be used initially depending on local expertise and individual characteristics with similar amputation free survival rates. However, not withstanding the high failure and re-intervention rate associated with angioplasty, patients who are expected to live for less than 1–2 years and have significant co-morbidity should probably, when possible, be offered angioplasty first, and subsequently proceed to bypass surgery if appropriate.[102]

Again aggressive multidisciplinary approach to foot disease associated with diabetes appears to save the lower extremity and be cost effective. The early aggressive control of infection and appropriate distal artery vascularisation allow orthopaedic, podiatric and reconstructive surgeons to perform foot-sparing surgery in patients with severe deformity and reduce amputation rates.

Novel Therapies.

There has been an increased body of work on novel therapies for the treatment of diabetic foot ulcers that are refractory to standard treatment. The therapies described below are adjunct treatments to the standard care previously outlined.

Inflammatory Mediators

It is possible to alter the wound healing process by supplementing diabetic wounds with inflammatory mediators. The use of recombinant tissue engineering allows for the large scale production of these inflammatory mediators in the clinical setting. The exact details of the inflammatory cascade have not been elucidated, but it is known that administration of these agents can benefit wound healing. Cytokines and growth factors have been investigated in diabetic ulceration.

Cytokines

Cytokines are small peptides and glycoproteins. They are produced by inflammatory cells and mediate the activities of haematopoietic cells. An example is granulocyte/macrophage colony-stimulating factor (GM-CSF). GM-CSF influences keratinocyte and fibroblast activity and increases the production of vascular endothelial growth factor and stimulates angiogenesis. It improves the function of neutrophils and this is pertinent in diabetes mellitus as there is abnormal chemotaxis, phagocytosis and intracellular killing. A meta-analysis revealed that GM-CSF did not hasten clinical healing of diabetic foot ulcers but did reduce amputation rates and other surgical procedures and should be considered in patients with limb threatening infections.[103]

Growth Factors

Growth factors are the most studied in wound healing as it is thought they have the potential for the most benefit in that they directly stimulate the cells involved in healing. They mediate non-haematopoetic cells e.g. fibroblasts and keratinocytes. The platelet-derived growth factors are the most studied. Recombinant platelet derived growth factor (rhPDGF) is licensed for the treatment of lower diabetic neuropathic ulcers that extend into the tissue or beyond and have an adequate blood supply (defined as $T_cPO_2 > 30$ mmHg). It is used as an adjunct to, and not a substitute for, good care practices including initial sharp, pressure relief and infection control. It is a topical gel used once a day for up to 20 weeks and covered with moist dressings.

A moderate increase in healing of diabetic foot ulcers at 20 weeks has been reported.[104] Previously published pivotal trials have shown that by the 20th week of care 35 percent more ulcers healed in the group randomized to receive rhPDGF than those who did not receive rhPDGF (i.e., a relative risk [RR] of about 1.35). This represents an estimate of the efficacy of rhPDGF under the tightly controlled conditions of randomised clinical trials. Margolis et al. studied 24,898 patients who had diabetic foot ulcers treated with becaplermin and

showed a relative risk of healing of 1.35 and a relative risk of amputation of 0.65 outside the clinical trial environment.[105]

A review by Bennett et al. concluded that growth factors may be useful in chronic, non-healing ulcers that do not respond to conventional care.[106] Growth factors including topical RGD (arginine glycine aspartic acid) peptide matrix may increase the rate of closure of diabetic foot ulcers.[107]

Tissue Engineered Biological Dressings

These new dressings are living tissue engineered products and were initially aimed to act as a skin substitute for ulcers. They are now thought to act by filling the wound with extra-cellular matrix and inducing the expression of growth factors and cytokines that contribute to wound healing.[108] The two products Dermagraft and Apligraft are approved in the USA.

Dermagraft, an allogenic living dermis equivalent is composed of neonatal fibroblasts from human foreskin cultured on a polyglactin scaffold. It is indicated for full thickness diabetic foot ulcers greater than 6 weeks duration, which extend through the dermis, but without tendon, muscle, joint or bone exposure. It should be used in conjunction with standard wound care regimens, in limbs with an adequate blood supply and is contra-indicated in ulcers with signs of infection. For this reason, it is not used as widely as the other available tissue engineered biological dressings. It must remain stored at -75 degrees Celsius until ready for use. One application which covers an area of 5.0 cm x 7.5 cm is usually required, although several trials have used up to 8 applications. Dermis derived from human fibroblast, (Dermograft) has been shown to increase wound healing rate. [109]

Apligraft is an allogeneic dermal equivalent derived from fibroblasts cultured in a contracted type I collagen matrix and an epidermis generated by keratinocytes. It is indicated in the treatment of full-thickness neuropathic diabetic foot ulcers of greater than three weeks duration, which have not responded to conventional ulcer therapy and extend through the dermis but without tendon, muscle, capsule

or bone exposure. It is also contraindicated in infection and the major adverse event is wound infection with its use. It may produce yellow exudates which can confuse with infection and its shelf life is 10 days. It has shown reduction in time to healing in diabetic foot ulcers. It is reported in the same study that complete wound healing was achieved in a considerable amount of patients after one or two applications and the clinician may defer a second application if there is a successful wound closure rate to indicate complete wound healing needing no further intervention.[108] One application can be tailored to the size to the ulcer it is stored in heat of 21 to 30 degrees Celsius.

Tissue engineered skin from cultured living dermis was shown to improve healing rate of ulcers and result in lower rates of osteomyelitis.[108] Although they are allogeneic, they do not stimulate antigenicity. Both Dermograft and Apligraft have shown efficacy in clinical trials. They may be used in addition to standard therapy and are limited by cost and require more clinical trials to ensure widespread use.

Stem Cells

There is an increasing body of research on the use of autologous stem cell administration to treat diabetic ulcers, ischaemic ulcers and critical limb ischaemia. A significant portion of patients, mostly with diabetes have peripheral vascular disease that is not amenable to revascularisation by either surgical bypass or endovascular stenting or balloon dilatation. This is due to the widespread nature of disease and the distal location of obstructions, in addition to the presence of multiple co-morbidities. The use of cell based therapies in these 'no option' patients may prevent amputation, The studies in the literature have enrolled a limited number of patients or represent case studies and cannot provide definitive proof of a therapeutic effect or report on long term safety.[110] The administration of the therapy has been intramuscular, intra-arterial, and topically. Bone marrow mononuclear cells and peripheral blood mononuclear cells have been studied.

Topical administration of autologous keratinocytes on a plasma polymerised carrier dressing has been reported to initiate wound healing in diabetic ulcers resistant to conventional therapy.[111] Yoshikawa et al. investigated the use of topically applied bone marrow-derived mesenchymal stem cells on a collagen sponge graft. This therapy caused wound healing in 18 of 20 patients.[112] Autologous biograft and mesenchymal stem cells have been shown to treat diabetic foot ulcers in case reports.[113] Autologous bone marrow stem cell transplantation with mononuclear stem cells was performed with intra-arterial and intramuscular administration resulting in ulcer healing and an increase in blood flow in human, as mentioned in the previous paragraph.[114]

Hyperbaric Oxygen Therapy

Hyperbaric Oxygen (HBO) therapy for treatment of diabetic ulcers has been investigated in the past. A review by Kranke et al. concluded that HBO significantly reduces the need for amputation and improves the chance of wound healing at one year.[115] It may be beneficial in addition to standard multidisciplinary management and may double the healing rate in non-ischaemic ulcers.[116] A review in Diabetes Care in 2000 stated a need for additional placebo controlled trials and pointed to the high cost of the intervention preventing its widespread use.[116] A review in 2008 concludes that more reliable clinical data are needed in order for HBO therapy to be recognised as an appropriate adjunct treatment for certain non-healing wounds.[117]

Other data is available on the use of maggots, acupuncture, negative pressure wound therapy, receptor for advanced glycation end product (RAGE) antibodies, connective tissue growth factor, Poly(ADP)-ribose polymerase (PARP) inhibitors, aldose reductase inhibitors and sorbitol dehydrogenase inhibitors.[118] The above are experimental treatments under investigation for the treatment of diabetic neuropathy and foot ulcers and are not recommended at the current time for standard treatment.

Conclusion

Diabetic Foot ulceration is a preventable disease. There have been suggestions that diabetic foot problems do not receive appropriate attention. Education and screening of diabetic feet is of utmost importance. Aggressive multidisciplinary management is of the utmost importance in treating and preventing foot ulcers and lowering the number of amputations and the financial and social cost as well as the detriment to the patient with diabetic neuropathy and foot ulceration. Newer therapies are coming online but are an adjunct to standard multidisciplinary management.

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Chapter 2.

Topical Stem and Progenitor Cell Therapy for Diabetic Foot Ulcers

Current treatment of diabetic foot ulceration is suboptimal. The biology of diabetic cutaneous ulcers is complex. Circulating angiogenic cell and mesenchymal stem cells are novel therapies which may correct the physiological defects in diabetic wound healing. The current data on circulating angiogenic cells and mesenchymal stem cells in treatment of diabetic foot ulceration is reviewed. A summary of the mechanism of action of cells with reference to the key preclinical and clinical studies investigating these cell types is presented. The benefit of topical delivery of cells using a biomaterial in comparison to other modes of cell transplantation is discussed. Clinical trials are required for the demonstration of clinical benefit of these novel treatments in humans. A review of the regulatory environment for undertaking a clinical trial using stem and progenitor cell therapy in humans is presented. There is potential for this therapy to treat other cutaneous diseases. Circulating angiogenic cell and mesenchymal stem cell therapy is an exciting new area of medicine with the potential to treat diseases which are otherwise sub-optimally managed.

Introduction

The prevalence of diabetes mellitus is increasing to epidemic proportions worldwide. Diabetic foot ulceration can affect up to 25 percent of people with diabetes mellitus throughout their lives. The most significant complication of foot ulceration is lower limb amputation, which arises from pre-existing ulcers in the majority of cases. Despite current clinical care protocols for ulcer treatment, there exists a high amputation rate. This presents a major burden for individual patients' health and well-being in addition to significant financial cost for health care systems. There is an urgent need for new medicinal products to treat diabetic ulcers. Cell-based therapies offer a novel treatment strategy to augment diabetic wound healing, increase ulcer healing rate and prevent amputation. The field of tissue engineering has developed commercially available skin substitutes for diabetic cutaneous wound repair. These products have incorporated somatic cells delivered in a bioengineered scaffold. However, having been available for the last decade, the majority have demonstrated only moderate clinical benefit in small clinical trials. In comparison, stem and progenitor cell therapy offer the potential for accelerated wound repair in addition to structural skin regeneration with functional recovery.

Stem cells have the ability to self-renew and differentiate into other cell types and are classified into adult stem and progenitor cells, embryonic stem cells and induced pluripotent stem cells. The mechanisms of action of stem and progenitor cells are not fully elucidated but include 1) differentiation to specialised cells e.g. skin cells of the dermis and epidermis 2) acting by paracrine or autocrine effects through the secretion of trophic factors e.g. the production of soluble mediators for neo-angiogenesis and 3) immuno-modulatory functions. Much research endeavour is determining the benefit of stem cell treatment on diabetic cutaneous wound healing with encouraging results in animal models. Regenerative medicine and tissue engineering specialties are rapidly elucidating the mechanisms of action of stem cells and translating the results of in-vitro and in-vivo experiments to human clinical trials. The requirements for success will be patient safety, clinical efficacy and convenience of use.

The focus of this chapter is to review the area of topical stem and progenitor cell therapy as a treatment for non-healing diabetic foot ulcers. It will focus on adult stem cells as these are nearer to use in human trials and do not pose the ethical constraints associated with the use of embryonic stem cells. Topical treatment with endothelial progenitor cell and mesenchymal stem cell therapy is presented in this review, and more specifically the delivery of these cells using biomaterial scaffolds. The currently available cell therapy products for wound repair will be presented. The case for adopting stem and progenitor cell therapy in research and treatment of diabetic foot ulcers will be discussed. The benefits of biomaterials and functionalised scaffolds for mediating cell therapy to a wound will be described. For both endothelial progenitor cells and mesenchymal stem cells, the potential mechanisms of action will be discussed with reference to key pre-clinical and clinical studies. The chapter will also describe strategies to enhance the therapeutic potential of stem and progenitor cells for wound healing. These will include the employment of matri-cellular proteins i.e. proteins associated with extracellular matrix that mediate diverse biological functions, gene therapy, conditioned media experiments and the delivery of several cell types. A section of the chapter will focus on translational of these advanced biological medicines to clinical trials. This includes issues regarding pre-clinical animal models, optimal cell source, safety and regulatory approval. Finally the chapter will highlight the potential of cell based therapies in other conditions causing cutaneous wounding, i.e burns, decubitus ulcers and other rare blistering conditions e.g. epidermolysis bullosa.

The Biology of Cutaneous Wounds

The repair of cutaneous wounds is a highly complex biological process. After injury, multiple biological pathways immediately become activated and are synchronised to respond.[1] Adult wound healing occurs by tissue repair with consequent scarring. The goal of adult wound healing is to repair a skin defect and to ensure the restoration of a barrier and to regain tensile strength. There is involvement of several cell types, cytokines and extra-cellular matrix components. The physiological overlapping pathways that are required for optimal wound healing include haemostasis (which occurs immediately on wounding) and inflammation with cell migration and proliferation (neutrophils

initially and subsequently macrophages). The proliferation of fibroblasts results in extra-cellular matrix deposition. Remodeling and wound contraction occur once closure of the wound takes place. Angiogenesis (growth of new blood vessels from pre-existing blood vessels) and re-epithelialisation are central processes in wound healing. This is a superficial description of wound healing and conveys the complexity of the process, but highlights the potential for disruption in a difficult to heal wound.[2,3] The physiological response to acute cutaneous wounds usually takes 3-14 days to complete.[4] Wound healing involves activation of keratinocytes, fibroblasts, endothelial cells, macrophages and platelets.[5]
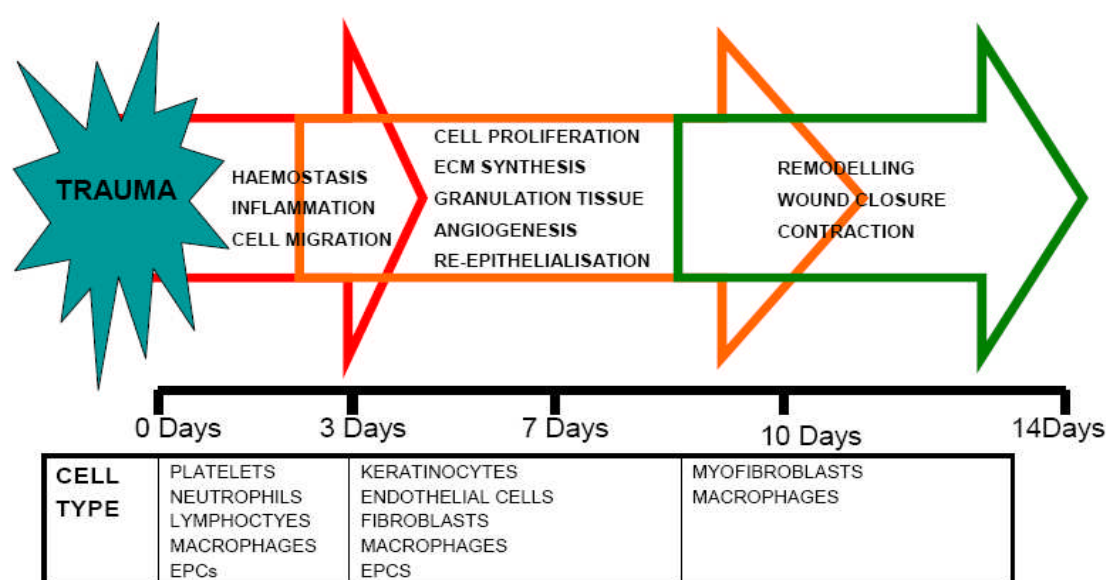
Figure I. Stages of normal wound healing with predominant cell types involved at each stage of process. The wound healing spectrum is a continuum with overlapping phases.

Diabetic Wound Healing

Delayed wound healing as occurs in diabetes mellitus results from dysregulation of the normal healings pathways. The diabetic wound is complex with contribution from infection, neuropathy and impaired vascular supply. There are many physiological defects in diabetic wounds. These include decreased or

43

impaired growth factor production, angiogenic response, macrophage function, collagen accumulation, epidermal barrier function, quantity of granulation tissue, keratinocyte and fibroblast migration and proliferation and bone healing. There is an imbalance between the accumulation of extra-cellular matrix components and their re-modelling by matrix metallo-proteinases.[5] In addition fibroblasts from diabetic wounds become senescent and show a decreased proliferative response to growth factors.[6] There is a chronic inflammatory environment associated with diabetic wounds. This is associated with a persistent increase in pro-inflammatory cytokine by various immune and non-immune cells and it is hypothesized that this blunts the acute, focused cytokine response needed to progress through the normal phases of wound healing.[7]

Angiogenesis and Wound Healing

The impaired vascular supply associated with diabetes leads to poor blood flow at the wound site impeding the optimal endogenous reparative response.[8] Impaired angiogenesis is a feature of diabetic wounds. In addition neovascularisation, or the de novo formation of new blood vessels is critical for granulation tissue formation and tissue regeneration in wound healing.[1] The impaired angiogenic response that occurs in diabetes mellitus leads to hypoxia at the wound site. Temporary hypoxia is requisite for normal wound healing. In the non-diabetic situation, hypoxia leads to activation of the transcription factor complex HIF-1α (Hypoxia inducible factor-1α), which leads to transcription of multiple genes required for successful wound healing. With diabetes, hyperglycaemia affects the stability and activation of HIF-1α. This suppresses platelet-derived growth factor, vascular endothelial growth factor and transforming growth factor-β, which are required for angiogenesis, in vitro and in vivo wound healing.[9]

Wound Repair versus Regeneration

Adult wound healing occurs by repair. Wound repair leads to scarring and results in decreased tensile strength of wounds. Skin regeneration is the regeneration of wounds with restoration of the normal function and anatomy of skin. In biology, foetal wound repair is a regenerative process, and some vertebrate species demonstrate successful tissue regeneration where the initial phase of wound

repair is followed by perfect structural and functional regeneration of the organ. An example of this is Xenopus limb regeneration. The challenge for scientists is to produce tissue engineered products that exhibit extra-cellular matrix re-modelling characteristics seen in embryonic wound repair to produce functional and durable skin.[10]

The Case for Novel Topically Applied Stem and Progenitor Cell Therapies

Burden of Diabetic Ulceration

There exists a growing global epidemic of diabetes mellitus. It is predicted that the prevalence of diabetes mellitus will be 4.4% of the global population or 366 million people by the year 2030.[11] In 2010, the prevalence of diabetes in China was reported as 9.7%.[12] This will likely continue to increase based on the prevalence of obesity in populations. Foot ulcers can affect 12 to 25 percent of persons with diabetes mellitus throughout their lives.[13] Lower limb disease is the most common source of complications and hospitalisation in the diabetic population.[14] Major lower limb amputations in patients with diabetes arise from preceding ulcers in 85% of cases.[15] The cost of treating diabetic foot ulcers creates a burden on healthcare resources. Boulton et al. reviewed the epidemiology and cost of treating foot ulceration globally and one report estimated the cost of diabetic foot ulceration treatment including amputation at €10.9 billion in the United States of America for the year 2001.[16] In addition to the cost to healthcare system budgets, for individual patients, the parameters of pain, social isolation, physical morbidity, restrictions in work capacity, and psychological well-being are negatively affected by leg ulceration.[17]

Classification of Diabetic Ulcers

Diabetic foot ulcers can be classified as ischaemic, neuropathic or neuro-ischaemic. The ability to heal ulcers is predicated on the restoration of an adequate blood supply. The typical angiographic pattern of ischaemic diabetic vasculopathy is occluded distal blood vessels. The optimal treatment of ischemic lower extremity ulcers is the restoration of blood flow. This review paper focuses on treatment of neuropathic ulcers. Neuropathic ulcers develop due to distal sensory loss and consequent foot deformity. Ulceration develops at sites of

excessive pressure predominantly under the first metatarsalphalangeal joint, in the majority due to unperceived trauma. Neuro-ischaemic ulcers are a combination of ischaemic and neuropathic ulcers.

Current Treatment Strategies

The management of the diabetic foot is complex requiring a multidisciplinary approach. A non-healing ulcer is an ulcer which has been present for > 8 weeks. Our group has reviewed the current standards of care required to investigate, treat and prevent diabetic foot ulceration and consequent amputation.[18] This manuscript highlights the benefit of routine examination and evaluation of the diabetic foot with identification of risk factors for ulceration. There are published risk stratification guidelines for diabetic foot ulceration based on the presence or absence of sensory loss, foot deformity and vascular insufficiency.[19] The current standard care involves removal of pressure from the ulcer, restoration of blood flow if peripheral vascular disease is present, debridement of the ulcer and institution of antibiotic therapy to control infection. Topical dressings, patient education, podiatry review, and orthotics are beneficial. A systematic review of the control arms of trials investigating novel treatments reported that for standard treatment of neuropathic diabetic ulcers, where blood supply had been adequate (as defined by a transcutaneous oxygen pressure of > 30 mmHg or an ankle-brachial index > 0.7), after 20 weeks 31% of diabetic neuropathic ulcers were healed and at 12 weeks, 24% of neuropathic ulcers were completely healed.[20] A protocol for the management of diabetic foot ulcers suggested treatment with growth factors and/or cellular therapy if wound healing is not is not observed after 2 weeks of standard therapy and a new epithelial layer has not formed.[21]

Benefit of a Cell-based Therapy for Non-healing Diabetic Ulcers.

It is evident that there is a critical clinical need to develop novel therapies for treatment of non-healing diabetic ulcers in order to prevent amputation and reduce the significant financial drain on healthcare budgets and burden on individuals health. The understanding of the patho-physiology of diabetic wound healing is important in the development of advanced wound healing treatments. It allows therapeutic targeting of the different phases of wound healing. Cell therapy may reverse the biological defects in diabetic wounds by acting as

reservoirs for cell and growth factor production. Gurtner *et al.* states that the ultimate solution to both under-healing and over-healing is likely to be administration of cells that retain the ability to elaborate the full complexity of biological signalling, together with the environmental cues that are needed to regulate the differentiation and proliferation of these cells.[1]

Limitations with Current Cell-based Therapy

To date clinical trials of topical cell based therapy for non-healing diabetic foot ulcers have yielded limited results. There are several reasons for this. One reason is methodological flaws in the clinical trials which have raised concerns over the validity of the results. Systematic reviews on skin replacement therapy have reported statistical benefit in wound healing endpoints. However there was a lack of information reported on safety, method of recruitment, randomization methods and blinding strategy for outcome assessments. There is a lack of power size calculations in some of the trials and little mention of dropouts in trial. The interventions did appear as safe as standard treatments.[22] It is felt that the deficiencies in clinical trials investigating skin replacement therapies for diabetic foot ulcers affect the conclusions of systematic reviews.[22-24] Further larger scale trials are required.

However the lack of clinical success with these advanced medicinal products is most likely not solely due to the aforementioned flaws in trial design. The current somatic cell therapy does not address the underlying pathology in the diabetic wound i.e. chronic inflammation and impaired angiogenesis. An efficient blood supply is central to normal wound healing, and delayed or inefficient angiogenesis will prolong ulceration and increase the probability of amputation. The current cell treatments do not target angiogenesis (blood vessel formation from pre-existing blood vessels) or neo-vasculogenesis (de novo blood vessel formation). Somatic cells do not differentiate into other cell types of the dermis and epidermis. The most frequently studied somatic cells include fibroblasts and keratinocytes. The employment of these cell treatments result in wound healing by repair and not by regeneration.

Potential Superiority of Treatment with Stem and Progenitor cells

Endothelial progenitor cells are a newly described cell type involved in angiogenesis. They can migrate to a site of injury/ischaemia and play a central role in vascular maintenance, angiogenesis and neo-vascularisation.[25] Adult mesenchymal stem treatment holds promise as this cell type addresses the key wound impairments seen in non-healing diabetic ulcers. They are immuno-modulatory and may create a more favourable inflammatory environment of the diabetic wound. They also promote angiogenesis by paracrine effects. Adult mesenchymal stem cells in diabetic wounds may in addition to beneficial paracrine activity, differentiate into other cell types e.g. epidermal keratincocytes, endothelial cells and pericytes in vivo.[26] In fact there is a growing body of evidence that the use of stem cells in wound healing in addition to augmenting wound repair, also promote skin regeneration and scarless wound healing.[27]

Endothelial Progenitor cells (EPCs)

Background

The discovery of putative EPCs by Asahara et al. in 1997[28] has illuminated the fields of vascular biology and diabetes related vascular dysfunction. For the first time, vasculogenesis or de novo blood vessel formation was determined to occur post-natally, as previously it was assumed to occur only during embryogenesis. The delivery of EPCs to ischaemic sites in the body offers the possibility of successful treatment of diabetic vascular disease. Worldwide, research groups are testing the hypothesis that EPC therapy may treat peripheral vascular disease and prevent the progression of non-healing diabetic foot ulcers to amputation. These cells are suitable for autologous therapy without immunological rejection but this approach may be hindered due to disease associated cell dysfunction.

EPC research is complicated by several issues. These include a lack of a standardised definition of the cell-type. The reports in the literature describe different identities, sources of isolation, culture methodologies and function. The cells maybe isolated from the peripheral blood, umbilical cord blood or bone

marrow. They are referred to as progenitor cells or stem cells. In a comprehensive review, Hirschi *et al.* describe three different EPC types isolated from mononuclear cells.[29] This classification reflects the different cell types reported as EPCs.

All three cell types are cultured in endothelial based media. The first cell type is named colony forming unit-Hill cells which arise from peripheral blood mononuclear cells which are non-adherent and give rise to a colony after 5 days in culture. The second cell type is a heterogenous collection of cells termed circulating angiogenic cells or early EPCs. These arise from mononuclear cells which are adherent to fibronectin or other matrix adhesion proteins after 4-7 days. They do not form colonies and have a low proliferative potential. They retain monocytic properties, secrete angiogenic factors and die after approximately 4 weeks in culture.[30] The third cell type is the endothelial colony forming cell or late EPC. These cells are derived from mononuclear cells that adhere to fibronectin and appear after 6-21 days. They display cobblestone morphology and from blood vessels in vitro. They are highly proliferative.[29] The cells maybe further characterised by their ability to ingest acetylated low density lipoprotein and bind *Ulex europaeusagglutinin 1* plant lectin. The different cell types may also be characterized by flow cytometry for surface immunophenotype. Late EPCs display marker CD 34, CD 133, VEGFR2, CD 31 and are negative for CD 45.

Benefit in Wound Healing

Topical and systemic EPC therapy is beneficial in wound healing. The predominant mechanism is the augmentation of angiogenesis and neo-vascularisation. Suh *et al.* reported that EPC therapy increased recruitment of monocytes and macrophages in addition to augmenting angiogenesis.[31] This highlights the benefit in early stages of wound healing. It is known that EPCs in wounds result in increased granulation tissue and wound closure.[32] It is intuitive that this is the case as a multitude of in vitro studies have shown the production of growth factors and cytokines from EPCs which are closely involved in wound healing. Table 1 presents the in vivo studies of EPC treatment for diabetic ulcers. These studies support the benefit of topical EPC therapy in diabetic wound

healing. The mechanism is reported as via paracrine effect, direct incorporation in blood vessels and differentiation into endothelial cells. The field of topical EPC therapy is in the early stages with benefit demonstrated in these studies. Intramuscular EPC therapy has shown benefit in critical limb ischaemia.[33] Further research is required to determine the benefit of EPCs delivered in a biomaterial. In addition the standardisation of cell dose, definition of cell type and animal model is required. The use of human cells in immunocompromised animals are required to further elucidate therapeutic efficacy

Mechanisms of Action

Paracrine Effect

Early EPCs and Late EPCs may contribute to post-natal neovascularisation by secretion of angiogenic cytokines and growth factors. The secretome of EPCs contains cytokines and growth factors which stimulate wound healing by increasing proliferation, migration and cell survival of the different cell types required for wound healing i.e. keratinocytes, endothelial cells and fibroblasts. The conditioned media from EPC cultures revealed production of interleukin-8, Stromal-derived factor-1α, vascular endothelial growth factor, platelet-derived growth factor and monocyte chemo-attractant protein-1.[34-36] These cytokines are central to cutaneous wound healing. Extensive secretome analysis can be undertaken using mass spectrometry to determine novel factors involved in EPC biology.[37]

Direct Incorporation in Blood Vessels

The second mechanism of action is the direct incorporation of EPCs into the growing blood vessel wall or the differentiation of these cells into mature endothelial cells. This mechanism is associated with late EPCs This mechanism has been shown in animal models and may not be as significant as the paracrine effect of cell therapy.[34] The comparison of EPC conditioned media as compared to EPC therapy alone for wound healing is important. The transplantation of conditioned media or identified therapeutic factors would allow for protein-based therapy. One study compared conditioned media from EPCs to EPC treatment alone in an animal model of cutaneous wound healing. Injection of EPC

conditioned media alone into the same diabetic wound in mice promoted wound healing and increased neovascularization to a similar extent as achieved with EPC transplantation alone.[38] However Marrotte et al. did not find similar therapeutic efficacy with less wound healing effect from EPC conditioned media.[25]

Impaired Angiogenesis in Diabetes due to EPC Dysfunction

It is known that EPCs are decreased in number and dysfunctional in people suffering from diabetes mellitus. The decrease in number of circulating EPCs in people with diabetes is still under investigation but defects in the SDF-1α/CXCR-4 pathway are becoming evident.[39] There are defects in EPC recruitment to wound sites. This is due to decreased mobilisation from the bone marrow and decreased homing to cutaneous wounds.[5] With diabetes there is decreased EPC participation in neoangiogenesis and neovsacularisation. Studies show that there are defects in cell migration, adhesion and tube formation.[40] There is also an increase in reactive oxygen species in EPCs from diabetes patients leading to cellular dysfunction. There is a body of evidence indicating that diabetes mellitus related EPC cell dysfunction represents a mechanism for impaired angiogenesis and impaired wound healing seen in diabetic patients.[25] The obstacle with autologus EPC therapy for diabetic complications is that there are a decreased number of cells available for transplantation. In addition, these autologous cells are dysfunctional.

Strategies to Increase Endothelial Progenitor Cell Efficacy

Topical Delivery

In normal healing, EPCs are released into the circulation from the bone marrow in response to ischaemia and travel to sites of tissue injury and participate in angiogenesis.[41] Diabetes-related vascular dysfunction arises from impairments in EPC mobilisation and homing to sites of ischaemia and cutaneous wounds. This has been shown in animal models of diabetic wound healing. In mice with cutaneous wounds and 4 weeks of streptozocin induced hyperglycaemia, the levels of circulating EPCs were unchanged but the levels of bone marrow derived EPCs within the wound granulation tissue were decreased as compared to non-

diabetic controls. The bone marrow derived EPCs from diabetic mice showed increased apoptosis and decreased proliferation in diabetic wound tissue as compared to non-diabetic controls.[42] The topical delivery of cells to a wound would overcome this homing defect and in addition would allow for ex-vivo manipulation during the cell isolation process. This ex-vivo manipulation may restore the EPC functional defect and succeed in restoring diabetic wound healing to the non-diabetic phenotype. Systemic delivery of stem cell results in cells being taken from the circulation in the lungs, spleen and liver and not reaching the wound.[43] The high prevalence of peripheral vascular disease in people with disease also inhibits the intravascular delivery of cell to the affect foot ulcer. The topical delivery of cells allows for concentrated doses of cells to be delivered to a skin wound and not become trapped in other sites in the body.

Matricellular Proteins: Osteopontin

Osteopontin (OPN) is a matricellular protein and is involved in tissue repair and angiogenesis. These proteins modulate cell function by interacting with cell-surface receptors, proteases, hormones, and other bioeffector molecules, as well as with structural matrix proteins such as collagens.[44] Decreased OPN is found in EPCs in diabetes mellitus. Dysfunction is reversed by exposure of EPCs to Osteopontin.[45] Osteopontin is involved in angiogenesis. Osteopontin knockout mice have decreased myocardial angiogenesis in response to ischaemia and delayed recovery after hindlimb ischaemia. OPN is involved in wound healing. Wound healing studies in osteopontin knockout mice show more residual debris and less matrix organisation than wildtype mice.[46] OPN expression is associated with enhanced angiogenesis and collagenisation of the wound bed. Delay in diabetic wound healing may be in part because of the low expression of OPN early in the wound bed after wounding, which may result in the poor migration of immune cells to the site of injury resulting in the accumulation of the cell debris, decreased recruitment of the endothelial cells, thus delay in angiogenesis and poor matrix organization.[47]

Biomaterials and Encapsulated Cells

Adhesion to a substrate allows transplanted cell survival over even short time frames, and manipulation of major cellular processes (e.g., migration,

proliferation, and differentiation) over longer time scales.[48] Sufficient numbers of cells do not remain in place when applied to the wound surface.[49] The use of biomaterials allows for more control in mediating delivery of cells to a wound. Current delivery options include injection of cells, delivery in extra-cellular matrix, delivery on a scaffold and delivery as part of a tissue engineering skin equivalents.[50] Silva *et al.* reported that delivery of EPCs using an alginate scaffold created a depot of endothelial progenitor cells which ensured sustained viability and function of cells in a mouse model of hind-limb ischaemia. This method was more successful than direct injection of cells alone. The vascular progenitor cells exit the biomaterial over time and repopulate damaged tissue and participate in the vascular network.[51] Cell encapsulation using biomaterials holds promise for both autologous and allogeneic cell therapy. The potential benefit of cell encapsulation with biomaterials includes sustained viability, the ability of the cell to avoid immune rejection, secrete therapeutic proteins and protect against mechanical stress.[52] Encapsulation of adult mesenchymal stem cells permits cell survival, proliferation and differentiation.[53]

Co-culture, Gene Therapy and Hyperoxia

It is hypothesised that endothelial progenitor cells act as angiogenic support cells by their paracrine activity. Co-administration of EPCs with smooth muscle progenitor cells increased vessel density in a mouse model of hind-limb ischaemia to a greater degree than administration of either cell alone.[54] Endothelial cells increase mesenchymal stem cell proliferation.[55] Gene therapy may rescue diabetic EPC dysfunction. Using an ex vivo gene transfer strategy, EPC cell cultures can serve as gene carriers and function as a temporal local production unit of de novo synthesized growth factors within the wound or skin replacement.[56] Increased reactive oxygen species and oxidative stress has been shown to give rise to the dysfunction of diabetic EPCs, leading to inhibition of cell proliferation, nitric oxide production, matrix metalloproteinase-9 activity and migration. Manganese superoxide dismutase gene therapy reverses this dysfunction restoring the cells ability to mediate angiogenesis and wound repair.[25] Hyperoxia increases nitric oxide mediated EPC activity.[57] The diabetes related dysfunction in hypoxia inducible factor which reduces vascular

endothelial growth factor production (required for EPC activity) can be reversed by topical wound administration the iron chelating agent desferoxamine.[58]

Increase Number of EPCs

Increasing EPC number for topical treatment increases the wound healing benefit of EPCs.[25] Granulocyte macrophage-colony stimulating factor (GM-CSF) increases monocyte derived peripheral blood EPCs. In-vitro animal studies reveal that proliferation of EPCs derived from the bone marrow can be accelerated by GM-CSF.[59] GM-CSF is routinely used in the patients receiving chemotherapy. It has been used in human clinical trial for investigation of autologous therapy in critical limb ischaemia.[33] In diabetic patients medications such as statin and angiotensin-converting enzyme inhibitor therapy can increase EPC number.[30]

Wound Model	EPC type	Delivery	Results	Mechanism	Ref.
Diabetic immuno-deficient mouse Ischemic ulcer	Human fetal CD133^{+} progenitor cells	Topical type 1 collagen seeded with EPCs	↑ wound closure ↑ angiogenesis	Paracrine signalling	35
Diabetic Mouse Full thickness ulcer	CD34^{+} EPCs	Intradermal injection	↑ wound closure. ↑epithelial coverage ↑ vascularisation	Not addressed	60
Diabetic Mouse full thickness ulcer	bone marrow derived CD34^{+} EPCs	Intradermal injection	↑vascularisation ↑wound closure	Paracrine signalling	61
Diabetic immuno-deficient mouse Full thickness ulcer	Human umbilical cord blood EPCs	Intradermal injection of EPCs and Topical EPC- CM	↑angiogenesis ↑ wound closure. Conditioned media showed therapeutically equivalent effect	Paracrine signalling	38
Genetically Diabetic mouse full thickness ulcer	Early EPCs	Topical delivery of genetically modified EPCs	↑wound closure ↑angiogenesis ↑ benefit with gene therapy and ↑ cell dose	Paracrine signaling EPCs present in capillaries	25
Diabetic mice full thickness cutaneous wounds	Lineage Negative progenitor cells (EPCs)	topically applied in a collagen scaffold	↑Wound Closure ↑ Vascular density	Differentiate into endothelial cells	62
Human diabetic critical limb ischaemia and foot ulceration	Autologous GM-CSF mobilized EPCs	Intra-muscular injections	Ulcer healing,	↑vessel density	33

Table I. Animal and human trials of EPC therapy for diabetic wounds

Mesenchymal Stem Cells (MSCs)

MSCs are adult fibroblast-like cells that differentiate along multiple mesenchymal pathways when exposed to appropriate stimuli. They adhere to tissue culture plastic and express cell surface markers for CD 105, CD 73, CD 90, and fail to express cell surface markers for CD 45, CD 34, CD 14, CD 11b, CD 79a and CD 19.[43] MSCs were originally isolated from bone marrow by Friedenstein *et al.* in 1968.[63] They may also be known as fibroblast colony forming units, marrow stromal cells, multipotent adult progenitor cells, connective tissue progenitor cells or multipotent mesenchymal stromal cells. MSCs may be found in almost all postnatal organs and tissues, including adipose, periosteum, synovial membrane, synovial fluid, muscle, dermis, deciduous teeth, pericytes, trabecular bone, infrapatellar fat pad, articular cartilage and umbilical cord blood.[64] Stem cells located outside of the bone marrow are generally referred to as "tissue stem cells". Tissue stem cells are located in sites called niches, which differ among various tissues e.g. a stem cell niche in the bulge area of hair follicles.[65]

MSC Treatment and Wound Healing

The complex pathology of diabetic foot ulceration requires that novel treatments are developed. The factors which are central to ongoing ulceration include poor blood supply, inflammation and decreased functioning of resident wound healing cells. MSC treatment has been shown to augment angiogenesis, suppress inflammation and augment wound healing cell functions. The focus of this review is the topical application of MSCs directly to the wound. There have been animal and human studies showing benefit of MSC therapy in the treatment of cutaneous wounds. Table 2 details the animal and human trials investigating topical MSC therapy in diabetic wounds. Topical MSC therapy is further advanced than EPC therapy. The in vivo studies in table 2 demonstrate that topical delivery of MSCs result in benefit in diabetic animal cutaneous wounds. It is clear that augmented wound repair occurs by differentiation of MSCs to cells with keratinocyte markers and paracrine mediated increases in angiogenesis and vessel density. Human studies although with a small number of patients have

shown benefit with several treatments. Further evidence is required from human cells in immunocompromised animal models to assess wound healing response. Standardisation in wound healing endpoints in both human and animal studies will allow comparison of effect between MSCs and modified MSCs. More research is required on the benefit of cells delivered using biomaterials.

Previous reports have investigated the benefit of topically applied fresh autologous bone marrow to wounds and have not been included in the table. In response to wounding and ischaemic conditions there is a mobilisation and homing of bone marrow MSCs to the wound. MSCs can undergo differentiation and act in a paracrine manner to reduce inflammation, stimulate angiogenesis and cause proliferation and migration of other cell types involved in wound healing. The MSC secretome is of central importance in realising the beneficial paracrine effects of the cells.

MSC: Mechanisms of Action

Differentiation

MSCs may differentiate into mesodermal tissue including osteocytes, chondrocytes and adipocytes. They can differentiate into several cell types including cardiomyocytes, vascular endothelial cells, neurons, hepatocytes and epithelial cells, making them a potential cell based treatment for human disease.[66] Allogeneic green fluorescent protein labelled bone marrow-derived MSCs have been applied directly to and injected around a cutaneous wound. MSC treatment accelerated wound closure, with increased re-epithelialisation, cellularity and angiogenesis. In the wound the MSCs expressed keratinocyte-specific protein keratin and formed glandular structures suggesting MSCs contribute to tissue regeneration by differentiating into keratinocytes.[26] MSCs differentiate into epidermal keratinocytes in vivo and in-vitro and also into skin appendages.[67,68]

Migration/Homing of MSCs

Bone marrow-derived MSCs contribute to cutaneous wound healing. The homing mechanisms are complex. Potential mechanisms include specific receptors or

ligands undergoing up-regulation in response to injury. This not only facilitates trafficking, adhesion and infiltration of MSCs but also provide MSCs with a specialised niche to support self-renewal and maintain pluripotency.[64] MSCs become arrested in blood vessels of injured or ischaemic tissues and secrete a variety of growth factors and cytokines beneficial for wound healing.[69]

Paracrine Effects of MSCs

MSCs act in a paracrine fashion to exert their beneficial effects. MSC-conditioned media medium augments wound repair with accelerated epithelialisation.[26] The analysis of MSC conditioned media revealed cytokines and growth factors required for wound healing. Vascular endothelial growth factor-a, Insulin like growth factor-1, epidermal growth factor, keratinocyte growth factor, angiopoietin-1, stromal derived factor-1, macrophage inflammatory protein-1 alpha and beta and erythropoietin were increased in MSC conditioned media when compared to dermal fibroblast conditioned media. Bone marrow-derived MSC conditioned medium attracts macrophages and endothelial progenitor cells to wounds.[70] MSC paracrine signaling has potential beneficial effects on angiogenesis, epithelialisation and fibro-proliferation during wound repair.[71] Wu *et al.* reported that BM-MSC treated diabetic wounds had increased capillary density, but the bone marrow-derived MSCs were not found in the new capillary structures. This paracrine effect was supported by analysis of the conditioned media which revealed high levels of VEGF-α and angiopoeitin-1 with increased endothelial tube formation.[26]

Immunomodulation

An important characteristic of MSCs is that they express low levels of major histocompatibility complex-I (MHC-I) molecules and do not express MHC-II molecules, CD 80, CD 40 or CD 86 on their cell surface.[72] This allows for allogeneic transplantation as MSCs. Human clinical trials have been conducted using allogeneic MSCs for the treatment of many conditions including graft-versus-host disease, type-1 diabetes and ischaemic heart disease, and neurological disorders e.g. stroke. MSCs possess immunosuppressive and anti-inflammatory properties in vitro and in vivo. They may suppress the proliferation and function of the innate and adaptive immune response and the

immunomodulatory functions may occur by direct cell-cell contact or by paracrine means.[72] Macrophages are a fundamental cell type in wound healing and immunity. They can be classified as having a pro-inflammatory M1 phenotype or polarisation and an anti-inflammatory M2 or wound healing phenotype. MSCs are capable of eliciting M2 polarisation of macrophages which contributes to marked acceleration of wound healing.[72]

Optimising MSC Therapeutic Effect

The high proliferation capacity of MSCs results in less dose limiting obstacles with MSC therapy. The allogeneic treatment allows for an "off-the-shelf" product. This is possible as the cells maybe cryopreserved for use in the future. MSCs are amenable to ex-vivo manipulation by gene therapy to provide cellular protection in an ischaemic environment.[73] Highly concentrated cell doses can be directly applied to the wound surface or adjacent to the wound and delivery can be mediated using biomaterials.[43] As is the case with EPCs, biomaterials ensure sustained viability of cells and cell encapsulation technology may protect cells from mechanical stress common in diabetic foot ulceration.[51,53] Table 2 summarises the published research, and includes studies showing the benefit of MSCs and manipulated MSCs on cutaneous wound healing. There is also a need to better understand the stem cell niche involved in diabetic cutaneous wounds. This is required as this niche is the necessary microenvironment to controlling stem cell fate. Tissue engineering should provide both cells and adequately functionalised biomaterials in order to restore the elements of the stem cell niche.[74]

Wound	MSC type	Delivery	Results	Mechanism	Ref.
Diabetic Mouse ulcers Human chronic ulcers DFU, n=1	Autologous Bone Marrow-Derived MSCs (BM-MSCs)	Topical Fibrin spray	↑ Wound Closure in mice and humans. No adverse events	↑ elastin fibres in MSC treated wound	49
Human chronic ulcers DFU, n=2	Autologous BM-MSCs	Collagen sponge with silicone film	Healing of wounds in 18 of 20 patients	↑ fibrous, fat and vascular tissue	75
Human DFU, n=1	Autologous BM-MSC	Fresh Bone marrow isolate applied to wound then covered with collagen seeded with MSCs	↓wound size with closing and healing of ulcer.	N/A	76
Human chronic wounds DFU, n=6	Autologous BM-MSCs + standard wound dressing	MSCs injected in and around ulcer, and ulcer covered by dressing	↓ ulcer size at 12 weeks	Increased inflammatory cells and capillary proliferation	77
Diabetic rats Full thickness wounds	BM-MSCs transfected with hepatocyte growth factor	Direct injection to wound dermis	Decreased wound healing time with adHGF MSCs	↑blood vessels ↓ collagen formation, ↓ AGEs with AdHGF MSCs	78
Diabetic mouse with full thickness ulcer	Allogeneic BM-MSCs	Topical application and injection around wound edge	↑wound closure ↑epithelia ↑cellularity ↑angiogenesis	Differentiate MSCs to keratinocytes Paracrine ↑angiogenesis	26
Diabetic mouse Full thickness ulcer	ATSC over-expressing SDF-1	Topical cell application to wound	↑ % wound closure ↓epithelial gap, ↑ cellularity	Differentiation and paracrine effect on wound cells	79
Diabetic Mouse Full thickness ulcer	Diabetic MSCs co-applied with14S,21 R -diHDHA	Topical MSCs applied to wound bed and injected intra-dermally	↑ epithelium ↑ GT	↑angiogenesis due to paracrine effect	80
Diabetic mouse Full thickness Ulcer	Umbilical cord-MSCs	Topical MSCs or systemic MSCs injection	↓ wound size with topically applied MSCs	TGF-β Paracrine effect	81
Diabetic mouse Full thickness	Autologous ATSC	Topical delivery using collagen scaffold	↑GT ↑epithelium ↑ no, capillary	Paracrine	82

ulcer					
Diabetic Mouse ulcer	Autologous BM-MSCs	Topical Delivery	↑epithelium ↑ GT ↑ blood vessels	Paracrine	83
DFU = Diabetic Foot Ulcer, BM = Bone Marrow, AGE = Advanced Glycation Endproducts ATSC = Adispose Tissue-derived stromal cells, GT = Granulation Tissue					

Table II. Animal and human trial on Topical MSC treatment of diabetic wounds

Biomaterial Scaffolds for Cell Therapy in Diabetic Wound Healing

Benefit of Cell Delivery using Scaffolds for Cell Therapy

As explained above, a limitation of systemic delivery of stem cells is the poor engraftment efficiency to the target site, specifically to the wound. It is known that cell infusions e.g. into ischaemic muscle, typically result in > 90% of cells rapidly dying.[50] Therefore some of the failures experienced in clinical cell transplantation may directly arise from the manner of administration of the cells rather than a lack of intrinsic bioactivity of the cells.[50] The use of a matrix is vital to the integrity of cell maintenance and growth because cells are anchorage dependent and require an appropriate milieu of mechanical strength, material support, controlled porosity and interconnected channelling.[84]

Determining the Optimal Biomaterial for Topical Treatment of Diabetic Wounds.

The goal of developing novel wound healing treatments is to reduce the time to complete wound closure and restore the barrier function of the skin. The ideal qualities of a skin substitute for diabetic ulcer wound repair is that it will be clinically effective, safe to the patient, inexpensive, easy to use, readily available, durable and encourage cell-matrix interactions. The ideal biomaterial should support reconstruction of new tissues without inflammation.[85]

There is a multitude of biomaterials for wound treatments commercially available and undergoing research. They may have different physicochemical profiles with differing mechanical and degradation properties. They may be synthetic or natural. Natural biomaterials are generally considered more

biocompatible and similar to the host extra-cellular matrix. The drawback of synthetic biomaterials is their lack of cellular recognition signals.[85] Skin substitutes can be classified based on **1.** anatomical structure (dermal, epidermal, dermo-epidermal), **2.** duration of cover (permanent, semi-permenant, temporary), **3.** type of biomaterial (biological: autologous, allogeneic, xenogeneic or synthetic: biodegradable, non-biodegradable), **4.** skin substitute composition (cellular, acellluar) and **5.** Where primary biomaterial loading with cellular components occurs (in vitro, in-vivo).[86] There are techniques used for development of tissue engineered ulcer healing products. These include 1. Transplantation of cells without matrix or scaffold, 2. Transplantation of biomaterials alone or with the addition of proteins e.g. cytokines and 3. Transplantation of cells in a 3-D scaffold.[87]

Currently Available Cell-based Biomaterial Dressings for Wound Healing

The focus of this chapter is on cell-based treatments using a 3-D scaffold. There are several terms that encompass such skin substitutes i.e. tissue-engineered skin, tissue engineered skin constructs, skin substitute bioconstructs, bioengineered skin, living skin replacements and living skin equivilants.[86] The gold standard skin replacement treatment for many conditions has been full-thickness skin grafting. There are inherent risks associated with autologous grafts e.g. donor site pain, scarring and infection or delayed healing and failure of graft at recipient site. The risks with non-autologous skin grafts include immune rejection and infection transmission.[88] A disadvantage of the currently available cell-based topical therapies is that they do not address the lack of angiogenic properties of the skin substitute. This is important as the successful ability of a skin graft to take to an ulcer is an adequate vascular supply. Table III summarises some of the commercially available skin substitutes and the clinical indications for their use. Apligraf and Dermagraf are temporary treatments for non-healing diabetic ulcers. These skin substitutes are biomaterials seeded with keratinocytes and/or fibroblasts. They are indicated as a topical treatment for non-healing diabetic ulcers in the USA.

Collagen as a Biomaterial

Collagen is the major extra-cellular matrix protein of the dermal layer of the skin. It forms an intrinsic part of blood vessels and supports angiogenesis. It is a commonly used biomaterial for topical cell based wound dressings e.g. Apligraf (Organogenesis). It displays low antigenicity with purification techniques available to eliminate the immunogenic telo-peptides.[85] Collagen is appropriate for temporary dressings as it is mechanically weak and undergoes degradation on implantation.[85] It is possible to manipulate collagen by cross-linking and enhance its physico-chemical properties. There are widely used commercial collagen based dressings for diabetic foot ulcers (e.g Promogram, which contains oxidised regenerated cellulose by Johnson & Johnson).[89] Integra (LifeSciences) is a wound healing product consisting of bovine type 1 collagen cross-linked with chondroitin-6-sulphate which is bonded to a silicone membrane. It acts as a template for fibroblast migration and capillary growth in vivo.[89] We have successfully seeded stem and progenitor cells in a collagen scaffold. Figure 2 is a scanning electron microscope image of EPCs and MSCs seeded in a collagen scaffold for 24 hours.

Product	Description	Indication
Apligraft /Graftskin Organogenesis Canton, MA, USA	Allogeneic neonatal foreskin keratinocytes and fibroblasts seeded in a type 1 bovine collagen	Diabetic foot ulcers venous leg ulcers Partial thickness burns Epidermolysis Bullosa
Dermagraft Advanced Biohealing Inc Lojalla, Ca, USA.	Allogeneic neonatal fibroblasts seeded in a polyglycolic acid (Dexon) or polyglactin-9-10Vicryl scaffold.	Full thickness DFU Epidermolysis Bullosa
TissueTech Autograft system. Laserskin and Hyalograft Fidia Farmaceutical Abano Terme Italy	Autologous fibroblasts and keratinocytes cultured on a hyaluronic acid laser perforated membrane	DFU and Chronic wounds
Epicel Genzyme Biosurgery Cambridge, MA, USA	Autologous keratinocytes and xenogenic proliferation-arrested mouse fibroblasts in petroleum gauze dressing	Full thickness burns burns taking >30% of body area
Transcyte Advanced Biohealing Inc, Lojolla California	Human allogeneic fibroblasts cultured on a nylon mesh pre-coated with collagen	Burns Transparent dressing
Orcel Ortec International New York ,NY USA	Type 1 Bovine collagen seeded with allogeneic neonatal fibroblasts and keratinocytes	donor sites for autografting, DFU Epidermolysis Bullosa
Epidex Modex Therapeutics Luzanne Switzerland	Cultured epidermal skin equivalent derived from keratinocyte precursors of human hair follicles	Chronic Leg uclers
Myskin Altrika Sheffield UK	Autologous keratinocytes grown on a silicone layer with irradiated murine fibroblasts	Non-healing wounds DFU, Burns, Pressure ulcers
Bioseed-S BioTissue Technologies Freiburg, Germany	Autologous keratinocytes resuspended in a fibrin sealant	Venous leg ulcers
Permaderm Regenicin www.regenicin.com	Autologous keratinocytes and fibroblasts seeded on collagen biomaterial	Burns Chronic Wounds
DFU = Diabetic foot ulcers		

Table III: Sample of currently available Cell-Scaffold skin replacement therapies and their indications
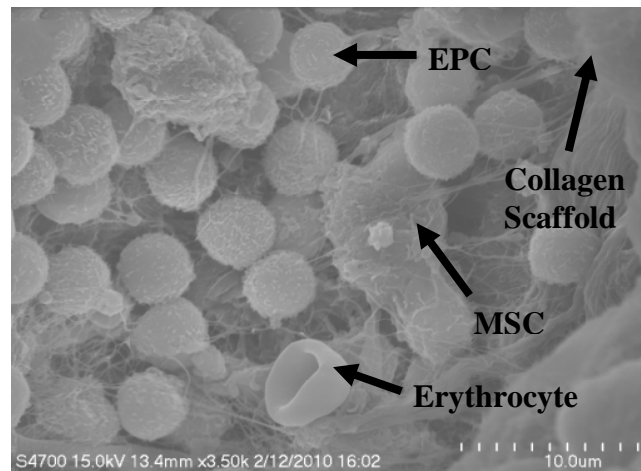
Figure II. Scanning electron microscope of co-culture of mesenchymal stem cells and early endothelial progenitor cells in a type 1 bovine collagen scaffold.

Translation to human therapy

Safety and Regulatory Approval

With any new cell-based therapy, it is mandatory to ensure safety for the patient. Any negative toxic side-effect of cell-based therapies would be a set back for the field of tissue engineering and regenerative medicine. In Europe, the European Medicines Agency (EMA) controls regulation and clinical trials of new cell based products. In North America, this process is under the remit of the Food and Drugs Administration (FDA). The EMA also advises on the development of stem cell products which are an example of an advanced therapy medicinal product (ATMP). In February 2011, the EMA published a document entitled "Reflection paper on stem cell-based medicinal products", highlighting the current situation in the field of stem cell therapy. (EMA 2011)   Safety and clinical efficacy is first proven by scientifically robust methodology in pre-clinical studies. It is required that the product is produced and clinical trials carried out according to international standards. These standards include GLP (good lab practice), GMP (good manufacturing practice), and GCP (Good clinical Practice). There is a requirement for quality checks in the manufacturing process. This includes analysis of cell treatment batches to ensure cell quality, identity, viability and traceability of cells. The goal is a robust, stringently controlled production and manufacturing process.

Preclinical Animal models: Choice of Model and Regulatory Issues

It is necessary to prove treatment efficacy in an animal model. An in vitro wound healing model is not sufficient to confirm treatment efficacy. The complexity of diabetic foot ulceration with its multi-factorial pathology cannot be realised in an animal model. There are over 10 different animal models of diabetic ulceration in the reported literature. There are inherent differences between animals and humans. These include cutaneous anatomy, vascular supply, duration of diabetes and the presence of other cardiovascular risk factor e.g. smoking.

In addition there are a myriad of endpoints reported in animal wound healing studies. The most robust clinically relevant wound healing endpoints are percentage wound closure and time to complete healing. The myriad of new treatment modalities under investigation have effects on different phases of the wound healing spectrum. The pig has skin felt to be the most close to humans but these are large expensive animals. The genetically modified, leptin receptor deficient diabetic mouse is widely used as a model of type 2 diabetes, but wound healing occurs by contraction in this model and does not reflect the human situation. The rabbit ear dermal ulcer model is a powerful model for examining re-epithelialisation and granulation tissue formation in an excisional wound.[2] A comprehensive review by Lammer's et al. recommends a more systematic evaluation of tissue-engineered constructs in animal models to enhance the comparison of different constructs, accelerating the trajectory to application in human patients.[90]

The EMA provides advice on the animal models to use for translation of cell-based therapy to humans. The choice of the most relevant animal model should be determined by the specific safety aspect to be evaluated. It advises the use of human cells to be tested in proof of concept and safety studies. This methodology requires the use of immuno-compromised models either genetically immuno-suppressed or treated with immuno-suppressants.[91] The persistence of cells and the functionality of the cells should be assessed. The potential of undifferentiated pluripotent stem cells to form tumours and be genetically unstable due to ex-vivo manipulation requires this to be assessed in animal models. This is more likely

with embryonic stem cells and pluripotent stem cells. Bio-distribution of cells to other organs and ectopic tissue formation need to be investigated. Prior to first-in-man studies, there are guidelines published by the EMA to identify and mitigate risks. Dose finding studies, immunological, pharmacokinetic, pharmco-dynamic and long term pharmaco-vigilant studies should be undertaken and planned.[91]

The use of biomaterials in conjunction with stem and progenitor cells is defined by the EMA as a 'tissue-engineered product' and falls under the term ATMP.[92] The experience with the development of allogeneic bi-layered skin has provided valuable experience on the development of skin replacement therapy. Apligraf (Organogenesis), a living bi-layered skin substitute has received approval from FDA. It is described as a Class III medical device via premarket approval and meets requirements for a human cell, tissue, and cellular and tissue-based product. As the product is made from viable human skin cells, it cannot be terminally sterilized, but safety concerns have been addressed. These include risk of transmission of infection, immunogenicity, immunological graft rejection and tumour formation. As cells are derived from neonatal foreskin, maternal blood of the neonatal donor and the cell banks are thoroughly screened for infectious agents, pathogens and other contaminants.[88,92]

Structured Diabetic Foot Care

Stem and progenitor cell-based topical treatments will not be used in isolation to treat diabetic foot ulceration. Ideally, these advanced biological treatments will be part of a treatment algorithm, which would see the implementation of standard care prior to use of cell therapy. If the restoration of vascular supply, removal of pressure, control of infection and debridement of the wound does not succeed in ulcer healing, then the indication for cell based therapy would apply. There are analyses of factors associated with lack of healing with fibroblast dermal substitutes. An episode of infection during 12 weeks of treatment was associated with a 3.4 times increased risk of non-closure of a wound.[88] High bacterial load in the wound negatively affects wound healing with Dermagraft and Browne et al. recommend reducing the bacterial load with combination antibiotics prior to the application of skin substitutes.[93] New treatment modalities are under

investigation which may augment wound healing and reduced bacterial load. Plasma therapy may reduce bacterial burden and enhance wound healing.[94]

Cost:Benefit Analysis

To ensure development of a successful topical cell based therapy, the product must have potential widespread use in the clinical arena. It must demonstrate clinical efficacy in clinical trials. In randomised controlled clinical trials the new product must show superiority both in comparison to standard care and to other market leaders in the field. It is expensive to conduct human clinical trials, therefore the product must demonstrate favourable health economics so as to be attractive to health care providers and industrial partners. To gain market access, manufacturers have to establish not only the efficacy of the product but also whether the product provides a cure at an acceptable cost per unit of health gain.[95] Several studies have investigated the cost-effectiveness of these products. The results feature favourable cost-effectiveness ratios in selected patient groups with chronic wounds. The cost of the product and product development should be offset against the total cost of care of the patient with non-healing diabetic foot ulcer.[95] There is a need for high quality clinical trials in this area.

Cell-based Therapies in other Dermatological Conditions

MSCs and EPCs have the potential to treat other dermatological conditions apart from diabetic foot ulceration. As seen in table III there are several conditions which may be suitable for these therapies including chronic venous and pressure ulcers, burns and epidermolysis bullosa. The economic burden of chronic wounds is potentially the largest burden on healthcare systems. Stem and progenitor cells may be used as orphan medications for life-threatening or extremely rare debilitating conditions. These drugs are not developed by large pharmaceuticals and are not subject to the same regulatory process. An example of this is the blistering disorder epidermolysis bullosa. In addition research into basic stem cell biology will elucidate mechanisms of action of stem cells which may guide the development of future therapies. The development of successful skin regeneration and elucidation of key molecules and biological systems will allow for scar free repair and increased strength of healed wounds. There are further exciting developments in the field of stem and progenitor cell therapy for

tissue regeneration. Hair follicle biology is important for skin biology and epidermal haemostasis. There are resident stem cells in the bulge area of the hair follicle which are required for re-epithelialisation during wound healing.[96] They are a readily isolatable source of adult stem cell suitable for autologous therapy.[97]

Conclusions

This book chapter has reviewed the current state of Stem and Progenitor cell therapy for non-healing diabetic foot ulceration. The urgent clinical need for developing improved novel cell treatments is stressed. The scientific basis for potential success with topical stem and progenitor therapy is reviewed. The advantage of using biomaterials to mediate cell delivery is discussed. Further developments in tissue engineering will provide more intelligent biomaterials which ensure better viability and control of stem cell fate and function. The logistical hurdles to translation of bench-side discoveries are reviewed and information provided on accelerated development of these advanced medicinal products.

The importance of translational science is being recognised as a key driver to the realisation of basic science discoveries for humans. There are strategic efforts to translate basic science to clinical benefit. This bench-to-bedside approach is the focus of government policies throughout the world with collaborations developing between pharmaceutical and biotechnology industries, academia and clinicians. The success of treatments will rely on clinical efficacy, safety, ease of use and cost-effectiveness. The potential to translate this technology to a variety of clinical dermatological disorders increases the attractiveness for industrial investment for further research and development of these products. A central component to the successful translation of this treatment will be the performance of robust randomised controlled trials. Stem cell therapy is a new field encompassing both tissue engineering and regenerative medicine science and holds promise for the improved treatment of diseases which are sub-optimally managed with current therapies.

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Chapter 3

Autologous Circulating Angiogenic cells treated with Osteopontin and Delivered via a Collagen Scaffold enhances wound healing in the Alloxan-induced Diabetic Rabbit Ear Ulcer Model.

Diabetic foot ulceration is the leading cause of amputation in people with diabetes mellitus. Peripheral vascular disease is present in the majority of patients with diabetic foot ulcers. Despite standard treatments there exists a high amputation rate. Circulating angiogenic cells previously known as early endothelial progenitor cells are derived from peripheral blood and support angiogenesis and vasculogenesis, providing a potential topical treatment for non-healing diabetic foot ulcers. Type 1 bovine collagen is a biomaterial which facilitates topical cell delivery to a diabetic wound. Osteopontin is a matricellular protein involved in wound healing and enhances the angiogenic potential of circulating angiogenic cells. A collagen scaffold seeded with circulating angiogenic cells was developed. Subsequently the effect of autologous circulating angiogenic cells seeded in a collagen scaffold and topically delivered to a diabetic cutaneous wound was assessed. The alloxan-induced diabetic rabbit ear ulcer model was used to determine healing in response to the following treatments: collagen seeded with autologous circulating angiogenic cells exposed to osteopontin, collagen seeded with autologous circulating angiogenic cells, collagen alone and untreated wound. Stereology was used to assess angiogenesis in wounds. The cells exposed to osteopontin and seeded on collagen increased percentage wound closure as compared to other groups. Increased angiogenesis was observed in the treatment with collagen and collagen seeded with circulating angiogenic cells.

Introduction

Diabetic foot ulceration is the most common reason for hospitalisation in people suffering from diabetes mellitus.[1] Despite conventional treatments, there exists a high amputation rate. Diabetes-related lower extremity amputations arise from pre-existing ulceration in approximately 85% of cases.[2] Topical cell-based therapy offers a new treatment for non-healing ulcers and may prevent the need for amputation. Normal cutaneous wound healing is a complex biological response to trauma, involving the sequential activation and integration of several biological processes. These include coagulation, inflammation, chemotaxis, angiogenesis and tissue remodelling. There are interactions of many different cell types and cytokines to allow normal wound healing. Delayed wound healing as occurs with diabetes mellitus results from a dysregulation of this process.

Endothelial progenitor cells (EPCs) are a recently discovered cell type which promote neoangiogenesis (new blood vessel formation arising from pre-existing blood vessels) and neovasculogenesis (de novo blood vessel formation).[3] Circulating angiogenic cells (CACs) have previously been described as early EPCs and are easily isolated from the mononuclear cell fraction of peripheral blood.[4] CACs have been shown to be involved in wound healing and are recruited to places of neovascularization in the granulation tissue, where they help release various cytokines that facilitate wound repair.[5] In the diabetic state, CACs are reduced in number and function and contribute to the poor wound healing response seen in diabetic ulceration.[6] Suh et al. demonstrated that transplanting EPCs into the wound increased recruitment of macrophages and revascularization, resulting in accelerated healing.[7]

CACs are known to be reduced in number and dysfunctional in people with diabetes mellitus.[8] A decreased expression of the matricellular protein osteopontin (OPN) is seen in CACs from people with diabetes mellitus. CAC dysfunction is reversed by exposure to OPN. The exposure of diabetic CACs to recombinant OPN augments angiogenesis in a mouse hind limb ischaemia model.[9] Autologous CACs can be manipulated ex vivo to augment cellular

function. This provides an attractive autologous topical therapy with CACs exposed to OPN for the treatment of diabetic ulceration

Conventional cell transplantation techniques using systemic intravenous injection or local intra-dermal injection results in low cell survival.[10,11] A collagen scaffold is an effective biomaterial to topically deliver cells to a wound and allows for sustained viability of cells in addition to maintaining cells at the wound site. Collagen is a constituent of the extra-cellular matrix and has been established for tissue engineering and cell therapy. It provides support for cell growth and attachment.

Three hypothesises were tested in this manuscript. **1**. Type 1 collagen is an effective biomaterial for topical delivery of CACs, **2.** The alloxan-induced diabetic rabbit ear ulcer model is a valid animal model of hyperglycaemic wound healing and **3**. Topical administration of autologous CACs exposed to OPN and applied to a hyperglycaemic full thickness cutaneous ulcer enhances wound healing.

Methodology

Isolation and Characterisation of Circulating Angiogenic Cells

Peripheral blood was isolated from healthy human volunteers. The study was approved by Galway University Hospital Ethics committee. Written informed consent was obtained. Approximately 30 mLs of peripheral blood was obtained using ethylenediaminetetraacetic (EDTA) coated bottles (BD Biosciences) and processed within 1 hour. CACs were isolated as previously described.[12] Briefly, the blood was mixed 1:1 with HANKS balanced salt solution (Sigma) and layered on Ficoll Paque-Plus (GE Healthcare). The sample was centrifuged and the buffy coat was removed to isolate the peripheral blood mononuclear cells. The cells were then washed once with red cell lysis buffer (Sigma), once with phosphate buffered saline (PBS) and re-suspended in EBM-2 media (Lonza). The media was supplemented with 2% fetal bovine serum, hydrocortisone, fibroblast growth factor, insulin-like growth factor-1, ascorbic acid epidermal growth factor

and GA-1000. Approximately 1 x 10^7 cells were plated per well on fibronectin coated 6 well plate (BD Bioscience). After 4 days, cells were trypsinised and viable cells were counted using trypan blue (Invitrogen) dye to count viable cells.

The cells were characterized by incorporation of DiI-acetylated-low-density lipoprotein (DiI-Ac-LDL) (Invitrogen). Briefly, the adherent cells were washed with PBS (Invitrogen) and incubated with 10ug/mL DiI-ac-LDL in 1ml of EBM-2 for 4 hours at 37°C. Cells were then washed with PBS and incubated at 37°C with fluorescein isothiocyanate (FITC)-conjugated *Ulex europaeus agglutinin* (FITC-lectin) (Invitrogen) at a concentration of 10μg/mL for 3 hours. Cells were photographed by an inverted fluorescence microscope (Olympus IX-71) and dual-stained cells were defined as CACs.

Collagen Extraction, Scaffold Formation and Cell Seeding

Type 1 bovine collagen solution was isolated and purified as described previously.[13] A collagen sponge was created by pipetting 1 mL of 3% (weight) type 1 bovine atellocollagen solution into 24 well tissue culture plates (Sarstedt Ltd). This was then lyophilized overnight using a VirTis freeze-dryer (Suffolk, U.K) The collagen sponge was prepared by washing once with HANKs balanced salt solution (Sigma), 3 washes with 70% ethanol, 2 washes of sterile water (Sigma), and 2 washes of supplemented EBM-2 (Lonza) media. After the washing steps the collagen scaffold was transferred to one well of a 48 well cell culture plate (Sarstedt). This was to ensure the scaffold was taking up all the base of the well. Cells were seeded by injecting 1 x 10^6 in 500 µl of EBM-2 using an insulin syringe (Becton, Dickinson and Company) and placed in an incubator for 24 hours in 37°C and 5% CO_2. The optimum time required for cell seeding on the scaffold was assessed by measuring metabolic activity of cells at 6, 12 and 24 hours after seeding

Assessment of cell viability and metabolic activity

The metabolic activity of cells was assessed using Alamar blue (resazurin, Invitrogen). Experiments were performed in triplicate. For human CAC experiments, using a dose of $1x10^6$ CACs, cells were washed once in Hanks balanced salt solution (Sigma) and incubated for 3 hours in 10% alamar blue. The absorbance of each sample was measured in a 96-well plate at wavelengths of 550 and 595nm using a microplate reader. The percentage of reduced alamar blue was determined as previously described.[14] For rabbit CACs, cells were washed once with Hanks balanced salt solution and incubated in alamar blue for 24 hours. The percentage reduction in alamar blue was assessed as above. This was repeated at 24, 48 and 72 hours. To assess live cells seeded in a collagen scaffold, calcein AM (Invitrogen) live stain was used. Briefly, cells seeded in scaffold as above and incubated with calcein live stain at concentration of 1:1000 for 30 minutes. Images were taken with confocal microscope (Zeiss LSM 510) at wavelengths 500-530 nm.

Processing of cells/tissue for scanning electron microscope

Samples were rinsed with 0.1Molar phosphate buffer, pH 7.2 and fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer for 2hrs at room temperature. The samples were dehydrated with ethanol and then placed in hexamethyldisilazane for 30 minutes. The samples were then gold-coated and analysed by scanning electron microscope (Hitachi S-4700)

In-vivo Model

18 male New Zealand white rabbits (3-3.5Kg) were used in the study. The protocol was approved by the ethics committee of the National University of Ireland, Galway and the study conducted under a license granted by the department of Health and Children, Dublin, Ireland. Rabbits were housed in individual cages and with a 12 hour light/dark cycle and controlled temperature and humidity. Rabbits were fed a standard chow and water *ad libitum*.

Induction of hyperglycaemia

16 rabbits were sedated with intramuscular injection of ketamine, xylazine and acepromazine. Hair was shaved off the back of the ears. Alloxan (150 mg/Kg) (Sigma-Aldrich) was made up in 30 mL of saline and administered via an ear vein using an intravenous cannula at a rate of 1.5 mL/min. After treatment water containing glucose was provided for 24 hours to prevent hypoglycemia in addition to provision of molasses to the animals' front paws to avoid hypoglycaemia. Blood glucose was checked daily from the marginal ear vein for the first week using Accucheck advantage strips (Roche). Blood sugars were checked once weekly once blood sugar stabilized. Insulin therapy was administered if the animal lost weight and had 'hi' glucose readings on glucometer (indicating blood glucose greater than 33 mmol/L), using insulin glargine (Sanofi-Aventis). 2 rabbits were not treated with alloxan and used as non-diabetic controls.

Autologous circulating angiogenic cell isolation and culture used for animal experiments.

Four weeks post-alloxan treatments, rabbits were anaesthetized using intramuscular acepromazine (0.1 mg/Kg) and inhaled isofluorane anaesthesia. 10 mL/Kg of blood was withdrawn from the marginal ear artery and collected in lithium heparin coated blood collection tubes (BD Biosciences). CACs were then isolated as previously described.[12] After 4 days EBM-2 media was changed and to half the cells, recombinant OPN (Sigma) was added at a concentration of 5µg/mL for 24 hours. Media was changed with no OPN added to the other half of CACs. Cells were trypsinised with 0.25X trypsin/EDTA (Sigma). Cell viability was assessed by trypan blue exclusion using a haemocytometer. 1×10^5 CACs was the maximum number of autologous EPCs which were able to be isolated from each animal. 5×10^4 CACs and 5×10^4 CACs exposed to OPN were seeded in a collagen scaffold for 24 hours as described above. The upper face of the CAC-seeded scaffold was applied to the base of the ulcers.

Surgical Procedure

Six days after phlebotomy, rabbits were anaesthetized using intramuscular injection of 0.1 mL/Kg xylazine and 0.12 mL/Kg of ketamine which is half dose analgesia. Sterile disposable 6 mm punch biopsies (Panvet, Ireland) were used to create 2 wounds on each ear. 60 wounds were made in 15 hyperglycaemic animals. The wounds were created and dermis exposed to bare cartilage. (Figure 1) Each wound was treated with one of four randomised treatment groups: untreated wounds, collagen scaffold alone, collagen scaffold seeded with 5×10^4 CACs, collagen scaffold seeded with 5×10^4 CACs exposed to OPN. The wounds were covered with polyurethane dressing (Opsite, Smith & Nephew) and the ear was stitched and covered with adhesive dressing, (Operfix, Promedicare) until day 7 (n=8). At 7 days rabbits were euthanized with intravenous sodium pentobarbital (2 mL). The same treatment groups were investigated in animals euthanized at 14 days (n=7). 20 ulcers were created in 2 non diabetic animals. 5 ulcers were created on each ear of the 2 non-diabetic animals. Wounds were covered using an adhesive dressing (Opsite). One hyperglycaemic animal was used to detect fluorescently labeled CACs in the wound. This animal was sacrificed at one week.
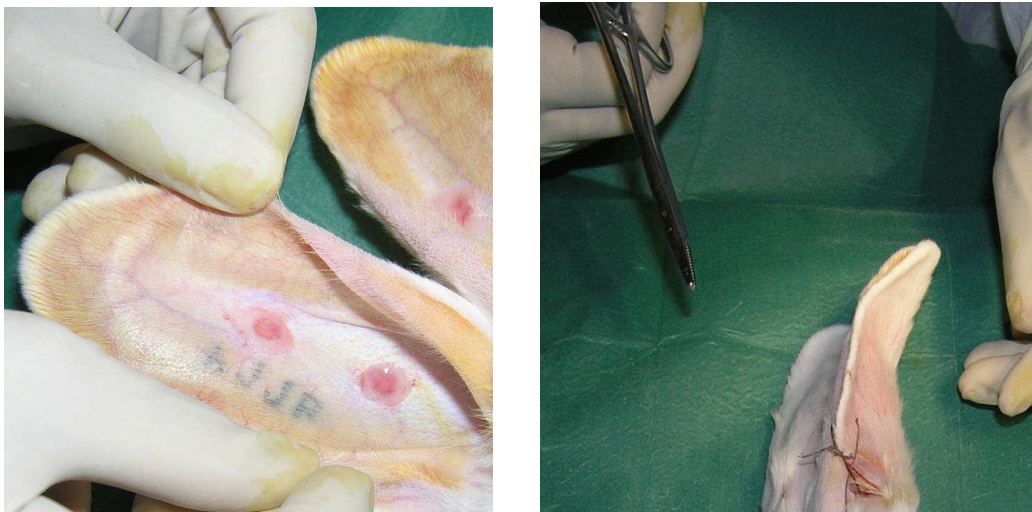
Figure 1. Wound creation and treatment application to rabbit ear to create 6mm full thickness ulcer. CAC seeded collagen scaffolds have been applied to the ulcers. Ear stitched to prevent dislodgement of treatment

Wound Closure

At necropsy ears were surgically removed and the wound area was traced. A fresh 6 mm wound was created. Wound closure was assessed using formula A. The area of the wound was determined by measuring the pixels within the tracing and this was assessed by Cell B software (Olympus). (Figure 2) Wound closure was assessed in both the diabetic and non-diabetic ulcers.
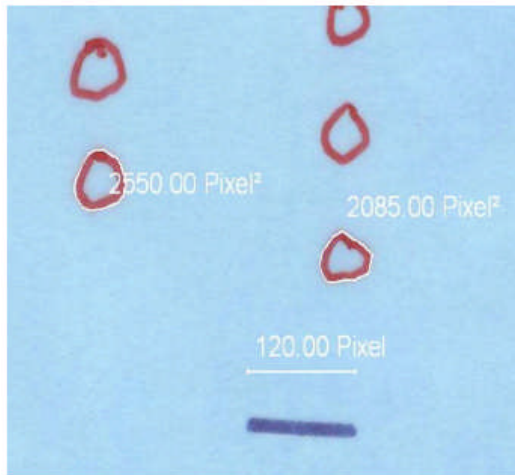
$$\%C = \frac{A_0 - A_i}{A_0} \times \frac{100}{1}$$

Figure 2   Formula A

Figure 2 Determining percentage wound closure from tracing. 10 mm = 120 pixels 120 pixels is divided into tracing of wound result to derive wound area in mm^2

Formula A. Percentage Wound Closure (%C) A_0 is the area of the wound at day 0 and A_i is the area of the wound at day 7 as measured by wound tracings.

Histology

The wounds were cut across the midline and fixed in 10% formalin for 24 hours. The tissue was processed and embedded in paraffin. 5 µm sections were taken when the tissue was reached. 9 sections were cut using a microtome every 150 µm into the wound for analysis. Three sections were placed on one slide. Sections were stained with haematoxylin and eosin using standard protocols.

Cell Labelling

CM-DiI (Invitrogen) was used to label fluorescently label cells for one animal experiment. Briefly, CACs were incubated with CM-DiI at a concentration of 4 µM for 20 minutes. The histological sections was obtained as above and the cells visualized using a fluorescent microscope in the TRITC-rhodamine channel

Wound Volume

Images were obtained at 2X magnification using an inverted microscope. (Olympus BX51) The thickness of the wound was measured from the top of the wound to the cartilage. (Figure 3) This was performed at 6 arbitrary points across the wound and an average of the 6 readings calculated. Cell B software was used for analysis. (Olympus) For each wound the average thickness of the wound was multiplied by the area obtained from the tracing of the wound at day of necropsy to calculate wound volume.
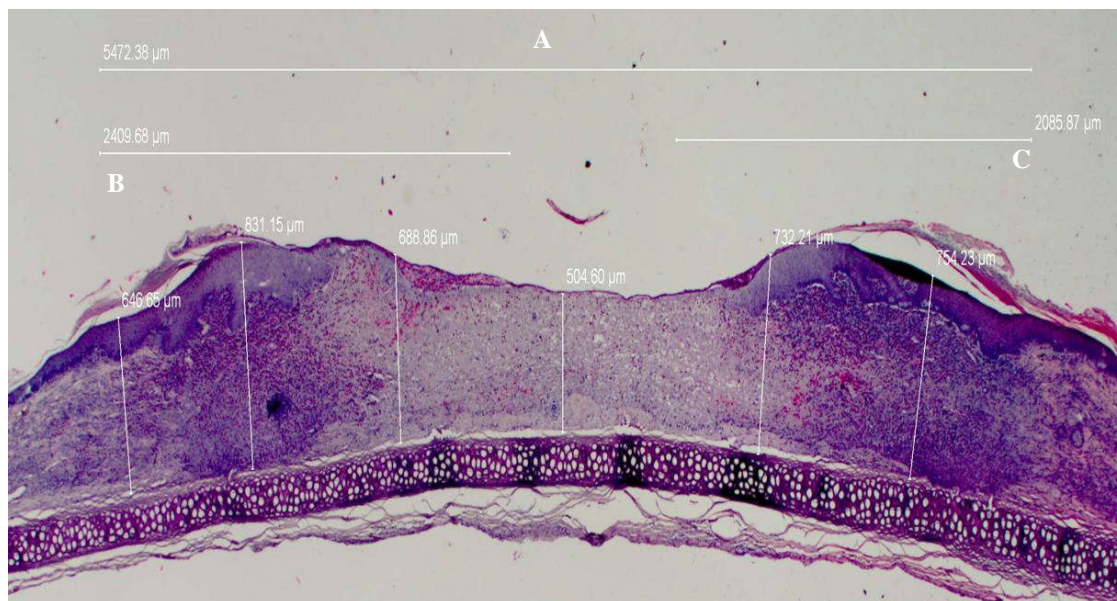
Figure 3. H+E section 1 week post treatment. Wound thickness was calculated by measuring 6 arbitrary wound thickness lengths taken from the cartilage to the wound surface and the average thickness obtained. Percentage epithelialisation is also measured (A,B,C). Image Magnification 2X

Epithelialisation

Epithelialisation was assessed by measuring the horizontal distance between the two wound edges (Figure 3 (A)). The wound edge was determined by a change in thickness of the epithelium, a lack of sebaceous glands, hair follicles and skin appendages. The length of the newly formed epithelium was measured (figure 3 (B+C)) and a percentage was obtained. This was performed on the first section analyzed from the centre of the wound.

Volume Fractions

The volume fraction of a feature within a particular reference space can be described as the proportion of space that the feature occupies in a unit volume.[15] Inflammatory cells were counted and included lymphocytes and neutrophils. This was counted using a 192 point grid using Image Pro Plus software (Media Cybernetics). (Figure 4) Neutrophils were identified as small dense circular multilobed cells and lymphocytes as small round dense cells with large nuclei. The volume fraction V_v is calculated as follows:

$$= \frac{P_P}{P_T}$$

Formula B: Volume fraction: P_P is the number of intersections of the grid on the cell and the P_T is the number of intersection on tissue. The volume fraction was multiplied by wound volume.
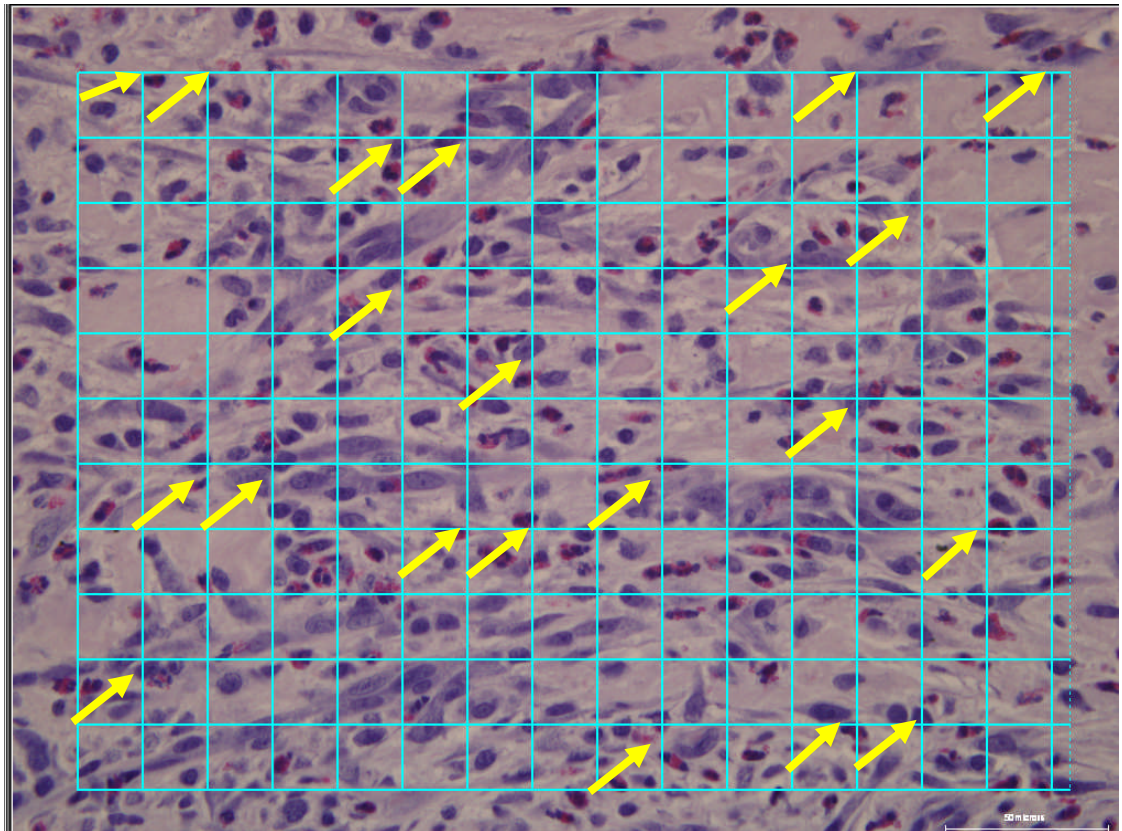
Figure 4. Calculating the volume fraction of inflammatory cells using a 192 point grid. Arrow indicates examples of inflammatory cells on intersection of the grid. (20X magnification.)

Stereology

Stereology is a means of assessing tissue responses to tissue constructs. It allows assessment of angiogenesis in vascular beds.[16] A series of cycloid lines were placed on the histology sections using image pro plus software (figure 5). In order to ensure the areas of the wound had the same chance of being selected, selection of the fields was done in a random manner. Five fields of view were obtained across the wound bed from one edge of the wound to the other edge.[15] The fields were captured at 20X magnification.

The parameters assessed were surface density of blood vessels, length density of blood vessels and radial diffusion distance between capillaries.[15,16] The surface density of blood vessels was calculated using Formula C and the length of test line was 2400nm. The surface area of blood vessels was then calculated by multiplying the surface density by wound volume. To calculate the length density

of blood vessels, a series of cycloid lines measuring 2240nm in length was rotated 90 degrees and placed on the histological section. The length density of blood vessels was calculated using Formula D. The total length of blood vessels in the wound was calculated by multiplying length density by wound volume. The radial diffusion distance was calculated using Formula E. The radial diffusion distance allows for the measurement of the distance between blood vessels, and is an indicator of the efficiency of a capillary network. The smaller the distance between blood vessels, the shorter distance required for nutrients to diffuse into surrounding tissues. Blood vessel diameter was calculated using Formula E.[17] The blood vessel diameter has been adjusted by multiplying by a shrinkage factor (1.6) that occurs with tissue processing. Analysis was carried out in a blinded manner.

$$S_V = 2 \times \frac{I}{L_T}$$

Formula C. Surface Density (S_V), I = Number of intersections with test line.

$$L_T = \text{Length of test line (2400 microns)}$$

$$L_V = \frac{(2 \times I_L)}{T_S}$$

Formula D. Length Density (L_V), I_L = Number of intersections with test line.

$$T_s = \text{thickness of the histological section (5 μm)}$$

$$R_{diff} = \frac{1}{\sqrt{\pi \times L_v}}.$$

Formula E. Radial Diffusion Distance (R_{diff}), L_V = Length Density.

$$d = \frac{S_V}{L_V \cdot \pi}$$

Formula F. Blood Vessel Diameter (d) S_V = Surface Density, L_V = Length Density.
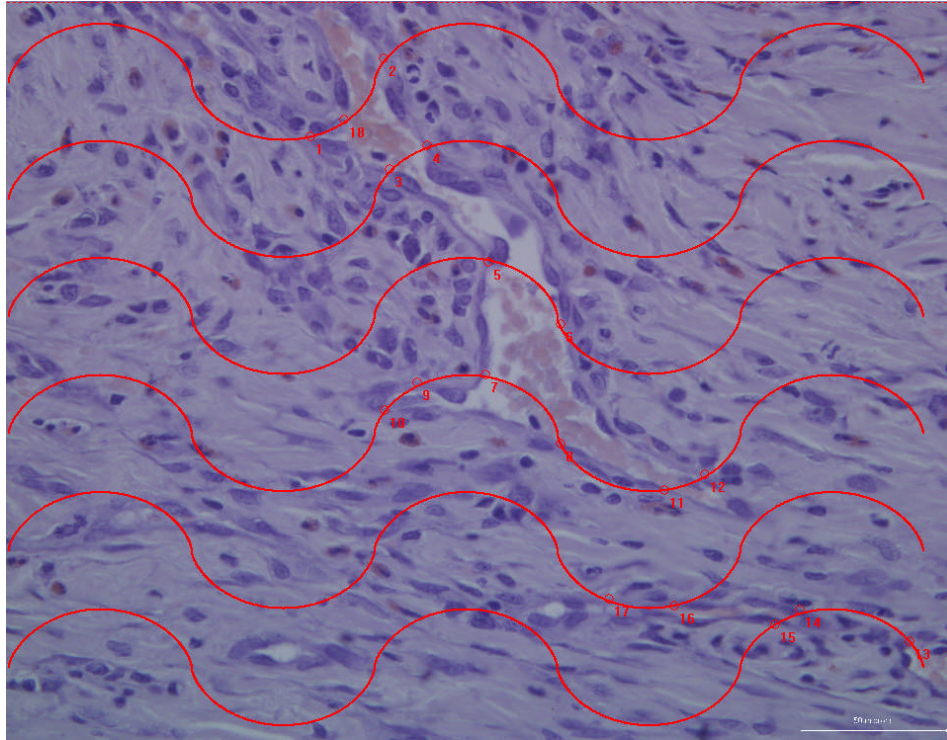
Figure 5: Enumerating blood vessels sterology. H+E section 20X magnification

Statistics.

All barcharts represent mean ± standard deviation. A 2 sample T-Test was used to assess the difference between healthy and diabetic wounds. One way ANOVA with Fisher's Pairwise comparisons was used to assess variance between treatment groups at 7 and 14 days post treatment. Minitab software was used to perform statistics.

Results

CAC characterization

CACs were characterized as demonstrated in figure 6. CACs are a heterogeneous cell population and are characterized by dual-staining for ac-LDL and lectin. These cells are mononuclear cells coated on fibronectin coated tissue culture plastic and cultured in endothelial-based media for 4 days.
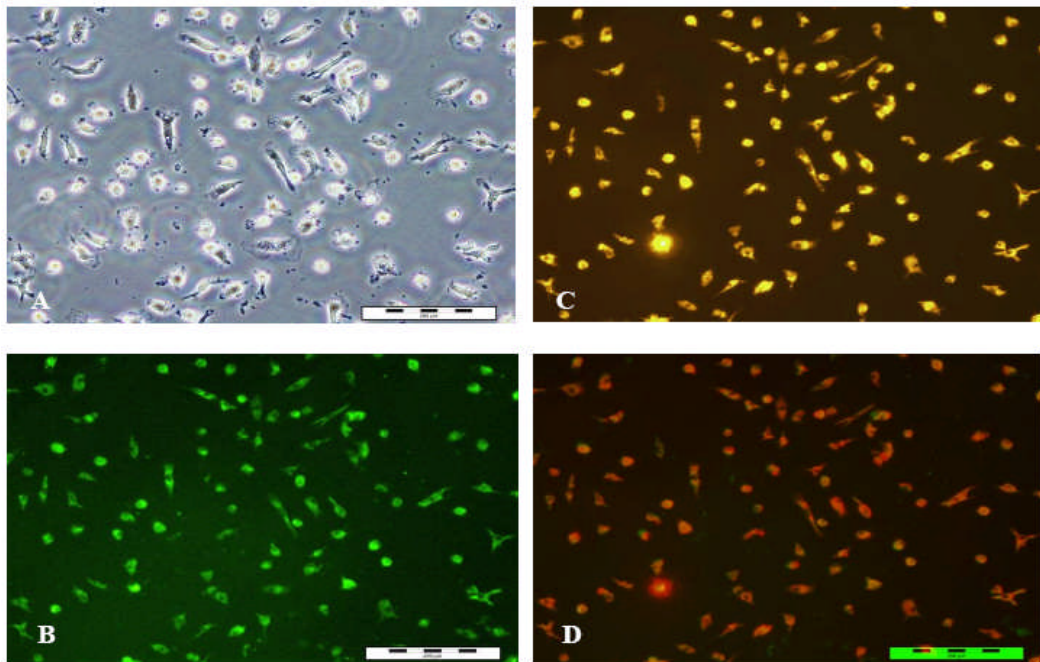
Figure 6. CAC characterization **A**. Bright field view **B.** FITC lectin (green) **C.** DiI-acetylated low density lipoprotein (red/red), **D.** Overlay of images of B and C.

Cell viability and Metabolic Activity

Human CACs retained 78% metabolic activity after seeding in a collagen scaffold for 24 hours when compared to cells seeded on tissue culture plastic. (Figure 7) CACs demonstrated increased metabolic activity 24 hours after seeding when compared to 6 hours. There were decreased cells in supernatant at 24 hours as compared to 12 hours. 24 hours was the duration of time the CACs were seeded prior to application to the diabetic ulcer. Calcaein stained viable

cells were evident on a collagen scaffold. CACs were viable and formed adhesions with the scaffold, as evident from scanning electron microscopy. (Figure 8A, 8B)
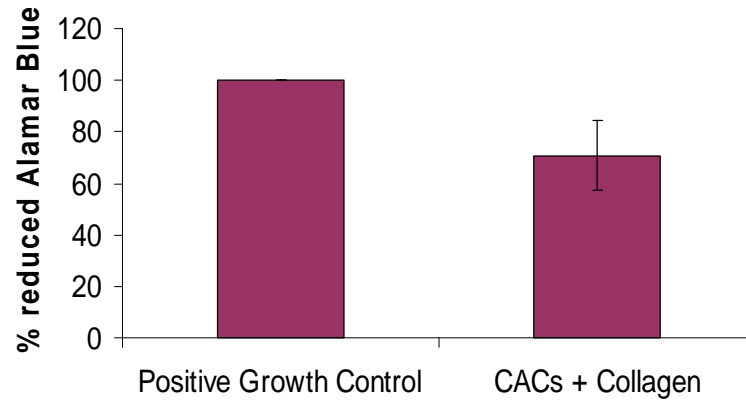
Figure 7: Viability after 24 hours of 1 x 10^6 CACs seeded on a type 1 bovine collagen scaffold.
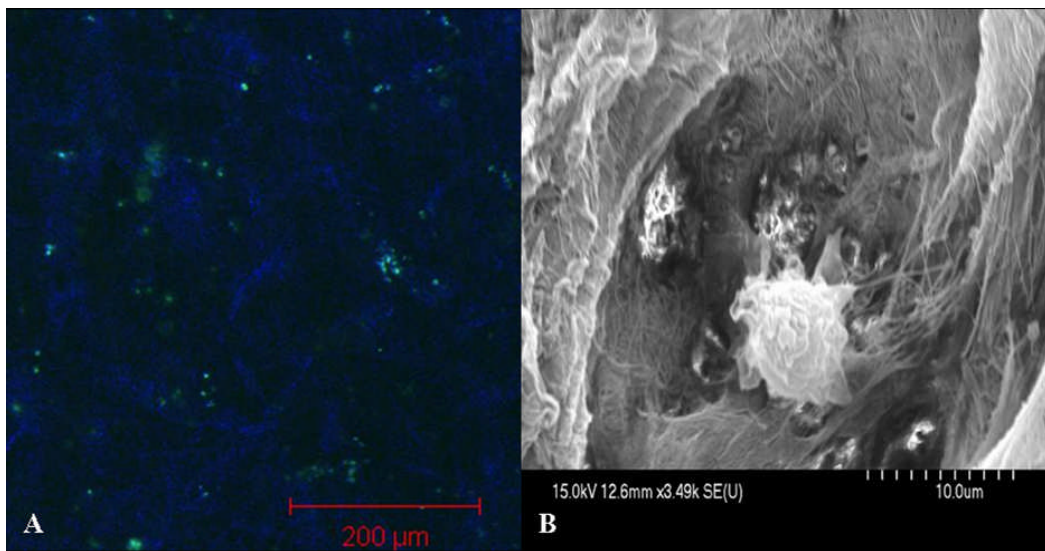
Figure 8: A. Calcein stained CACs 24 hours after seeding in a type 1 bovine collagen scaffold. Green stain is calcein positive live cells and blue is collagen scaffold. **B.** Scanning electron microscope image of CACs 24 hours after seeding in a collagen scaffold.

In-Vivo model

Animals became hyperglycaemic within 48 hours and remained hyperglycemic for the duration of the study (figure 9). Insulin therapy was required for three animals. Eight animals lost weight after alloxan administration ranging from 0.1 to 0.5 Kg. 2 animals died due to hyperglycaemia and are not included in the study.
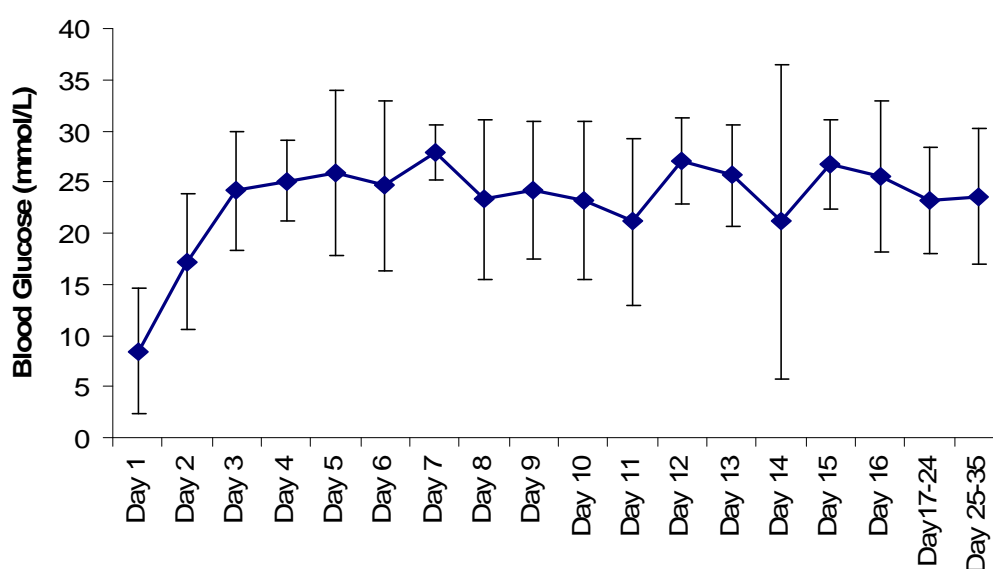
Figure 9. Mean glucose of animals over 35 day period. Blood glucose readings available recorded. (Error bar = Standard Deviation)

Wound Closure in Diabetic and Non-diabetic Animals

Wound Closure is calculated as in Formula A and Figure 2. Representative gross images of wounds are presented in figure 10. Diabetic animals had significantly reduced percentage wound closure at 1 week as compared to non-diabetic animals (figure 11).
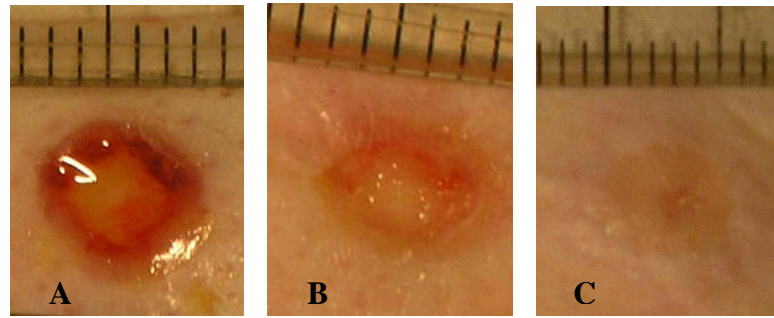
Figure 10. Representative gross pictures of wounds. **A**. Diabetic wound after 1 week. **B**. Non diabetic wound after 1 week. **C**. Control wound in diabetic animals after 2 weeks.
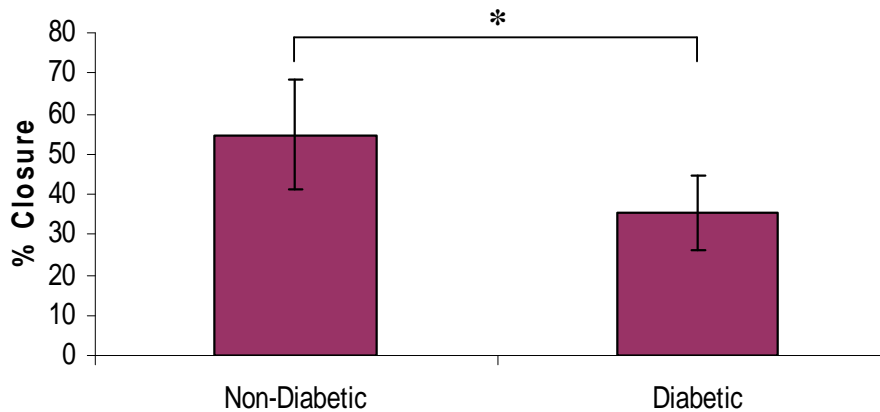
Figure 11. Percentage wound closure of non-diabetic and diabetic wounds 1 week after wounding. (*P<0.05, analysed by 2 sample t test)

Cell Labelling and Metabolic Activity of Rabbit CACs.

5×10^4 rabbit CACs demonstrated metabolic activity 24 hours after seeding in a collagen scaffold. This reduced to the same level as collagen control at 96 hours (figure 12). Labeled CAC were present in wound 1 week after treatment. The cells were located at the wound edge and in proximity to cartilage (figure 13). Figure 14 demonstrates examples of H +E sections CACs seeded in a collagen scaffold prior to application to the wound.
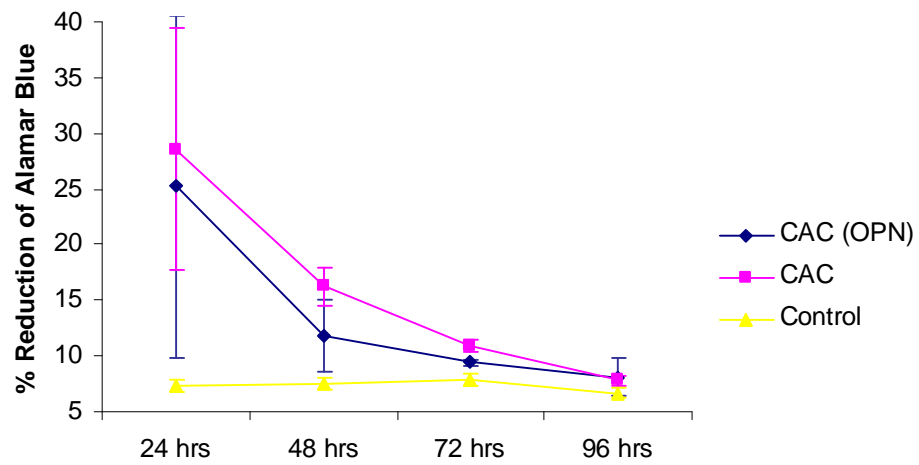
Figure 12. Time course of Metabolic Activity of 5 x 10^4 hyperglycemic rabbit CACs seeded on a collagen scaffold. (n=3) Metabolic activity was reduced to the same level as control at 96 hours post seeding in in vtro.
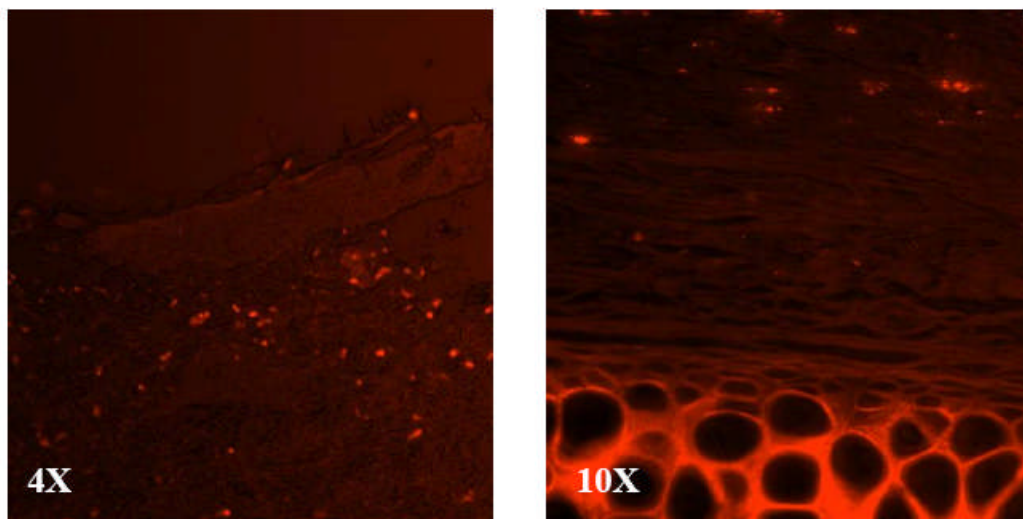

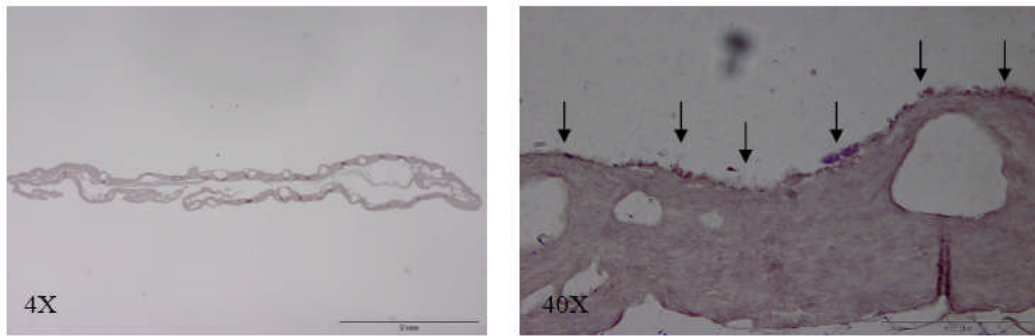**Figure 13**. CM-DiI labelled CACs. Fluorescently labelled CACs in wound one week post treatment.

Figure 14. H+E section of rabbit CACs seeded in a collagen scaffold. CACs located in the surface of the scaffold (arrows)

Percentage Wound Closure in Treatment Groups

Representative pictures of wounds are shown in figure 15. The treatment group with CACs exposed to OPN and delivered using a collagen scaffold demonstrated increased percentage wound closure at one week post treatment as compared to CACs delivered in a collagen scaffold, collagen alone and untreated wound (figure 16).

There was no difference between treatment groups and untreated wounds at 14 days. The percentage wound closure ($\pm$ SD) for untreated wound, collagen alone, collagen seeded with CACs and collagen seeded with CACs exposed to OPN was 86.3%($\pm$6.4), 87.3%($\pm$9.9), 91%($\pm$3.1) and 91.8%($\pm$4.4) respectively. At 7 and 14 days post treatment, there was no significant difference in percentage epithelialization between groups. At the 2 week time-point, 6 of the 7 control wounds were completely epithelialised.
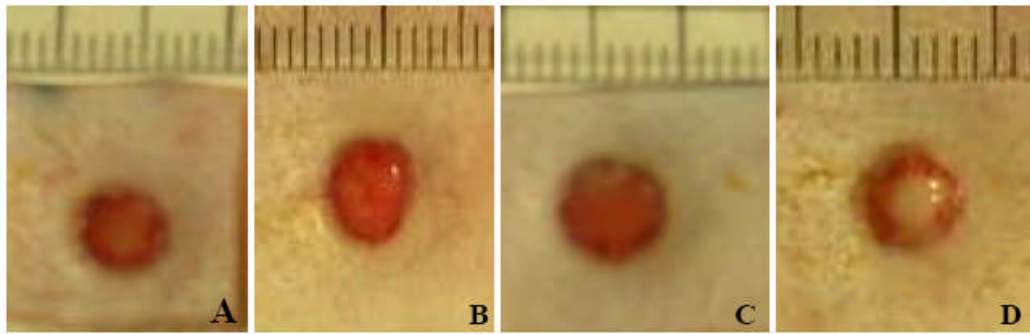
Figure 15. Representative gross pictures diabetic ulcers 1 week after treatment. **A.** CACs exposed to osteopontin and seeded on a collagen scaffold. **B.** CACs seeded in a collagen scaffold. **C.** Collagen treatment. **D.** Untreated Wound.
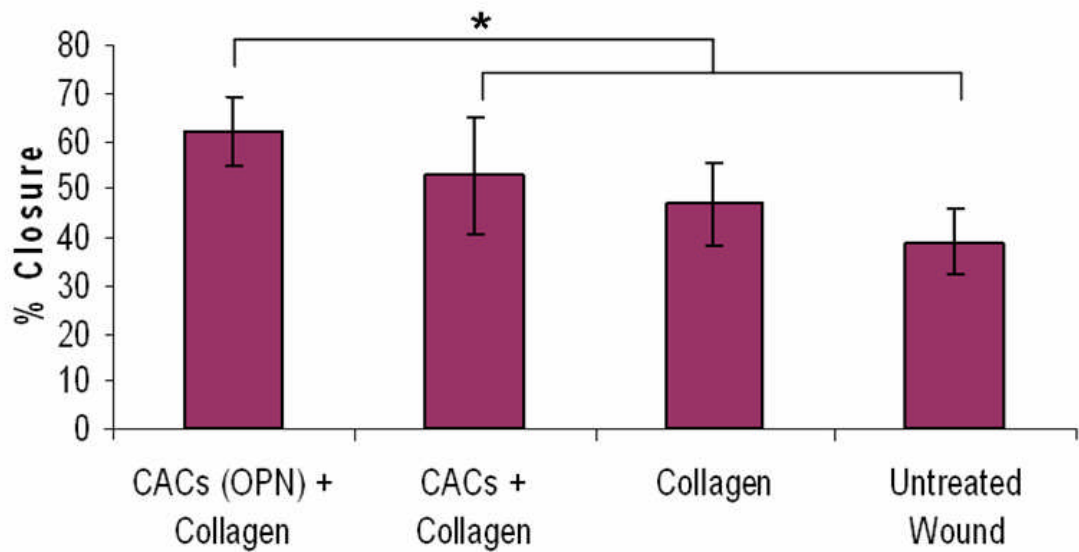

Figure 16. Percentage wound closures as assessed using wound tracing. (Analysed by ANOVA followed by Fishers Pairwise comparison, n=8, *$P<0.05$). The percentage wound closure at 1 week is significantly greater in the CACs (OPN) group when compared to CACs + Collagen, Collagen alone and Untreated wound. CAC(OPN) =CACs exposed to osteopontin and seeded on a collagen scaffold.

Wound Volume, Stereology and Inflammation

At 1 week post treatment, collagen alone has a higher wound volume, as compared to CACs exposed to OPN and seeded in a collagen scaffold and untreated wound. This increased wound volume seen with collagen results in an increased length of blood vessels in the collagen treated wound and increased surface area of blood vessels in the collagen treated wound. (Table 1) A significantly increased surface density and length density, in addition to a reduced radial diffusion distance is noted with all treatment groups in comparison to untreated wound. Blood vessels in the wounds treated with CACs exposed to OPN have a significantly larger diameter than untreated wound.

Analyses of inflammatory cell infiltrate revealed a significant difference between the 3 treatment groups as compared to untreated wound. The wounds treated with CACs exposed to OPN and delivered in a collagen scaffold revealed a trend towards reduced inflammatory cell infiltrate as compared to wounds treated with CACs and seeded in collagen and collagen alone (See Table 1).

Parameter	CACs (OPN) + Collagen	CACs + Collagen	Collagen	Untreated
Wound Volume (mm^3)	13.2±5.1*	14.1±3.5	17.4±3.6[#]*	12.03±3.69
Volume of Inflammatory Cells (mm^3)	2.37±0.97[#]	3.01±1.16[#]	3.21±0.91[#]	1.39±0.5
Surface Density of blood vessels in wound (mm^{-1})	22.4±9.3[#]	21.6±4.34[#]	25.6±5.8[#]	11.1 ± 5
Surface Area of Blood Vessels (mm^2)	306.3±178	330.5±20[#]	442±112[#]	146±116
Length Density of Blood Vessels in Wound (mm^{-2})	6212±1424[#]	6534±1589[#]	7575±1610[#]	4116±1093
Total Length of blood vessels in wound (mm)	81678±35117	90490± 26997[#]	131220± 34187[#]	49337±51595
Radial Diffusion Distance (µm)	7.29±0.9[#]	7.16±0.1[#]	6.56±0.6[#]	9.05±0.15
Vessel Diameter (µm)	1.8±0.47*	1.76±0.46	1.74±0.3	1.35±0.38*

Table 1. Stereological analysis of wounds in diabetic animals. (Analysed by ANOVA followed by Fishers pairwise comparison, n=8, *P<0.05, [#]P<0.05 compared to untreated wound.

Discussion

Diabetic foot ulceration leads to amputation in a significant proportion of cases. There is a critical need to create novel treatments to prevent amputation in addition to decreasing time to wound closure. EPCs delivered either systemically or topically have been shown to augment wound healing in both diabetic and non-diabetic cutaneous wound models.[6,7] Early EPCs or CACs are decreased in number and are dysfunctional in people with diabetes.[18] Our group has shown that the exposure of CACs to OPN rescues the angiogenic potential of diabetic CACs and restores blood flow in hindlimb ischemia.[9]

Cells topically delivered to wounds do not remain at the wound site.[19] Type 1 bovine collagen is a biomaterial that is involved in wound healing and effectively mediates topical cell delivery to a cutaneous wound. In this study human CACs were successfully seeded in a bovine type I collagen scaffold. Metabolic activity was 78% at 24 hours. (Figure 7) CACs were viable and formed cytoplamic connections with the scaffold, as evident from calcein staining and scanning electron microscopy (figure 8).

There is no standard animal model to study diabetic ulceration. The impaired cutaneous wound healing associated with diabetes mellitus in humans maybe due to a large number of factors.[20] It is not possible to fully replicate these factors in animal models. The hyperglycaemic rabbit ear ulcer is similar to the human diabetic foot ulcer as it heals by epithelialisation and granulation tissue formation. The rabbit ear ulcer does not heal by skin contraction. This is advantageous over cutaneous wounds in rodents which heal by contraction thus contrasting with the human condition. The rabbit ear skin is a specialized organ. It has a unique blood supply. We have previously described the use of this model Breen.[15] However, this is the first time that this model has been utilized to investigate topical cell therapy as treatment of cutaneous ulcers and allows for the assessment of the effect of transplantation of autologous ex-vivo modified CACs. After 5 weeks of hyperglycemia, wounds were created. One week after wound creation, percentage wound closure was less in the diabetic animals as compared to non-diabetic animal. These data validate that the animal model

represents a model of compromised wound healing. In diabetic animals, control wounds appear completely healed at 14 days post ulcer creation. (figure 10c) At 14 days 86% of wounds are completely epithelialised. The 2 week time-point was not investigated further due to the near complete wound healing response seen in control wounds at 2 weeks. However, at 7 days there was evidence of impaired wound healing in this diabetic model and this time point was used to assess the effect of topical cell delivery.

Fluorescently labeled rabbit CACs seeded in a collagen scaffold were identified in the wound 1 week after treatment. The metabolic activity of 5×10^4 CACs reduced to control levels by 96 hours in vitro. In addition CACs do not proliferate in culture and die after approximately 3-4 weeks when cultured on fibronectin-coated tissue culture plastic.[8]

Topical CAC therapy was investigated on wounds created after 5 weeks of hyperglycemia. Autologous CACs were successfully isolated from peripheral blood of hyperglycemic rabbits and cells were exposed to OPN ex-vivo. They were then topically re-administered to a full thickness cutaneous ulcer via a collagen scaffold. The treatment group with CACs exposed to OPN and delivered using a collagen scaffold demonstrated increased percentage wound closure at one week post treatment as compared to CACs delivered in a collagen scaffold, collagen alone and untreated wounds. Percentage wound closure is a clinically relevant endpoint in wound healing research and a wound that closes more quickly is a goal for wound healing treatment efficacy.

Stereology is a robust scientific tool for determining tissue responses to tissue engineered constructs in vivo. An extensive analysis was performed through one half of each wound at 150 micrometer intervals. The angiogenic response to the wound treatments was assessed using stereology. This beneficial increase in percentage wound closure is not fully explained by an increase in the angiogenesis endpoints of length density and surface density of blood vessels. For surface density and length density, all treatment groups were significantly different from untreated wound. The increased wound volume seen with collagen results in an increased length of blood vessels in the collagen treated wound and

increased surface area of blood vessels (Table 1). A significantly increased surface density and length density, in addition to a reduced radial diffusion distance is noted with all treatment groups in comparison to untreated wound. It can be concluded that angiogenesis is supported by the three treatment groups. This is in keeping with previous observations that type 1 collagen is known to support angiogenesis.[21] As the current methodology is an extensive analysis through the wound this is regarded as a representative sample from wounds. Blood vessels in the wounds treated with CACs exposed to OPN have a significantly larger diameter than untreated wounds. This suggests a more accelerated wound healing response with more mature blood vessels.

Analyses carried out for inflammatory cell infiltrate revealed a significant difference observed between the 3 treatment groups as compared to untreated wound. The wounds treated with CACs exposed to OPN and delivered in a collagen scaffold revealed a trend to a reduced inflammatory cell infiltrate as compared to wounds treated with CACs and seeded in collagen and collagen alone. This result highlights a potentially reduced inflammatory environment in wounds treated with CACs exposed to OPN. This is beneficial as non-healing diabetic ulcers are associated with an increased inflammatory cell environment.[22] Others have reported an increase in macrophage and monocyte infiltration into the wound after EPC treatment. Furthermore, an increase in granulation tissue of the wounds treated with EPCs has been reported.[5,7]

OPN has been shown to rescue diabetes-related CAC dysfunction.[9] We have demonstrated an effective autologous topical CAC therapy in a model of diabetic ulceration. This allows for successful ex vivo modification of CACs. This is clinically relevant to the human situation. The endpoint of percentage wound closure which is clinically meaningful is achieved with CACs exposed to OPN and delivered using a collagen scaffold. The mechanism is not fully elucidated as OPN is a molecule with diverse biological functions. The biology of diabetic wounds is highly complex with greater than 100 physiological defects.[22] The endpoint of percentage wound closure provides evidence of efficacy, however elucidation of the exact mechanism deserves further research.

In conclusion an animal model of impaired wound healing in diabetes is described. Subsequently a scaffold based cell transfer system is demonstrated. This treatment results in better healing with OPN modified CACs on collagen. It is shown that all collagen groups had increased angiogenesis.

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Chapter 4

In vitro analysis of the secretome of diabetic and non-diabetic circulating angiogenic cells: a pilot study

Diabetes-related vascular dysfunction is a leading cause of mortality and morbidity. Endothelial progenitor cells (EPCs) otherwise known as circulating angiogenic cells (CACs) promote neoangiogenesis and neovasculogenesis. The angiogenic effect of CACs may occur through paracrine means. CACs are known to be dysfunctional in people with diabetes. In order to further investigate this, the conditioned media of CACs from 6 people with type 1 diabetes mellitus and 6 healthy controls was compared. In vitro analysis was performed using matrigel assays and angiogenesis-related protein assessment. The conditioned media from diabetic CACs demonstrated significantly decreased angiogenic potential as evidenced from decreased tubule formation with the matrigel assay. Five angiogenesis-related proteins were detected in the conditioned media of both diabetic and non-diabetic CACs. These included CXCL-4, CXCL-16, matrix metalloprotein-9, tissue inhibitor of metalloproteinase-1 and plasminogen activator inhibitor-1. There was no significant difference between angiogenic related-protein levels observed in diabetic and non-diabetic CAC conditioned media.

Introduction

Endothelial progenitor cells are recently discovered cell type that promotes neoangiogenesis and neo-vascularisation.[1] Prior to this, neo-vascularisation or de novo new blood formation was felt to occur only during embryogenesis. Endothelial progenitor cells (EPCs) are classified into early EPCs or circulating angiogenic cells (CACs) and late EPCs. This classification is based on culture methodology, cell morphology and staining with fluorescently labeled acetylated low density lipoprotein and lectin. Further characterization may be performed with surface immuno-phenotyping.[2] CACs promote angiogenesis through paracrine mechanisms.[3] Diabetes is associated with an increased incidence of micro- and macro-vascular complications. There is a decreased number of circulating EPCs in people with diabetes. In addition diabetic EPCs and normal EPCs exposed to high glucose have display impaired function.[4] It is postulated that diabetes-related vascular dysfunction arises from the reduced number and function associated with diabetic circulating angiogenic cells.

It is hypothesised that the impaired CAC mediated vasculogenesis associated with diabetes is due to differences in paracrine effects of CACs. To further investigate this, an vitro analysis of the angiogenic potential of CACs was performed. In addition we performed a detailed analysis of angiogenesis-related proteins secreted from CACs.

Methods

Study Participants

The study was approved by Galway university hospital research ethics committee. Written informed consent was obtained from diabetic patients and non-diabetic healthy controls. Inclusion criteria included people with type 1 diabetes and no micro- or macro-vascular complications, a HbA1c > 8%, no complications of diabetes, non-smokers, not on any medication except for insulin therapy, with normal blood pressure, lipids and normal serum creatinine. The patients were recruited from the diabetes clinic University College Hospital,

Galway. Control subjects were recruited from non-diabetic age and sex matched healthy controls.

Cell Culture

30 mls of peripheral blood was obtained from the ante-cubital fossa in ethylenediaminetetraacetic acid (EDTA) coated bottles (BD Biosciences). CACs were isolated as previously described.[5] Briefly, blood was mixed 1:1 with HANKS balanced salt solution (Sigma). After ficoll density centrifugation, the buffy coat was removed and the mononuclear cell fraction was washed with red cell lysis buffer (Sigma), phosphate buffered saline (PBS, Sigma) and resuspended in EBM-2 media (Lonza). The media was supplemented with 2% fetal bovine serum, hydrocortisone, fibroblast growth factor, insulin-like growth factor-1, ascorbic acid epidermal growth factor and GA-1000. 5×10^6 peripheral blood mononuclear cells were counted using trypan blue exclusion dye (Sigma) and plated on fibronectin coated tissue plates. The cells were cultured for 4 days in EBM-2. The cells were then washed and 1 mL of foetal bovine serum free and growth factor free EBM-2 media (Lonza) was added to the well. After 48 hours, the media was collected and centrifuged at 14,000 rcf. A 48 hour time point was used as the 48 hour time point resulted in increased amount of angiogenesis-related protein as compared to 24 hours. The supernatant was removed and frozen at -20°C for further analysis.

Cell Characterisation

Cells were characterized by staining with 1,1-dilinoleyl-3,3,3',3' tetramethylindocarbocyanine perchlorate (DiI)-labelled acetylated-low-density lipoprotein (Dil-acLDL; Invitrogen, Oregeon, USA) and fluorecein isothiocyanate (FITC)-conjugated Ulex Europaeus Agglutinin lectin (FITC-UAE-lectin). Briefly, adherent cells were washed with PBS and incubated with 10µg/mL DiI-acLDL in 1ml of EBM-2 for 4 hours at 37ºC. Cells were then washed with PBS and incubated at 37ºC with FITC-conjugated UEA (Invitrogen) at a concentration of 10µg/mL for 3 hours. Finally, images were obtained using fluorescence inverted microscopy (Olympus IX-71).

Matrigel Assay

The matrigel assay is an assay of in vitro angiogenic potential. It is based on the ability of endothelial cells to form tubules when added to matrigel.[6] 27 wells of a 48 well plate were coated with 100 µl of growth factor reduced matrigel (BD Biosciences) and allowed to polymerise for 20 minutes at 37°C. 3 x 10^4 passage 4 human umbilical vein endothelial cells (HUVECs) were suspended in 100 µl of growth factor and foetal bovine serum supplemented EBM-2. The cell suspension was added to the matrigel coated wells. 100 µL of conditioned media was added to these HUVECs and slides were incubated at 37ºC and 5% CO_2 for 16 hours. The experiment was performed in triplicate from 3 separate diabetic donors and 3 non-diabetic controls. The control group included 100µl of growth factor free and foetal bovine serum free EBM-2 media.

For quantification of tubules, 5 random fields were taken at 10X magnification using Olympus IX71 inverted microscope from each well of the wells. The total pixel density of tubules was calculated using analysis D imaging system software (Olympus). The total pixel density of tubules in the diabetic group was compared to the non-diabetic group using a two-tailed t test. Results were expressed as mean +/- standard deviation.

Assessment of Cell Viability and Metabolic Activity

The metabolic activity of cells was assessed using Alamar blue or rezaurin (Invitrogen). Resazurin is a non-toxic calorimetric dye that changes colour from blue to red with oxidation and reduction reaction in mitochondria. The percentage of reduced Alamar blue was determined as previously described.[7] 24 hours after seeding, the cells were washed once in HANKS balanced salt solution (Sigma) and incubated for 3 hours in 10% alamar blue. The absorbance of each sample was measured in a 96-well plate at wavelengths of 550 and 595nm using a microplate reader. The amount of fluorescence produced is proportional to the number of living cells.

Angiogenesis Antibody Array

A human angiogenesis proteome profiler array kit (Invitrogen) was used to determine relative amounts of angiogenesis-related protein simultaneously in the conditioned media of cells. This permits detection of 55 angiogenesis-related proteins in cell medium. The conditioned media is mixed with a cocktail of biotinylated detection antibodies and incubated with a nitrocellulose membrane which contains capture antibodies. The antibody/protein mixture binds to the membrane. Using strepavidin-HRP and chemi-luminescent detection reagents, light is produced at each spot in proportion to the amount of analyte bound. This is quantified by densitometry using Image J software. A comparison of the level of proteins in both diabetic and non-diabetic conditioned media is obtained. Results are expressed as mean densitometry ± standard deviation.

Statistics

Difference between groups was assessed using a two-tailed t-test, with significance taken as P< 0.05. Barcharts represent mean ± standard deviation

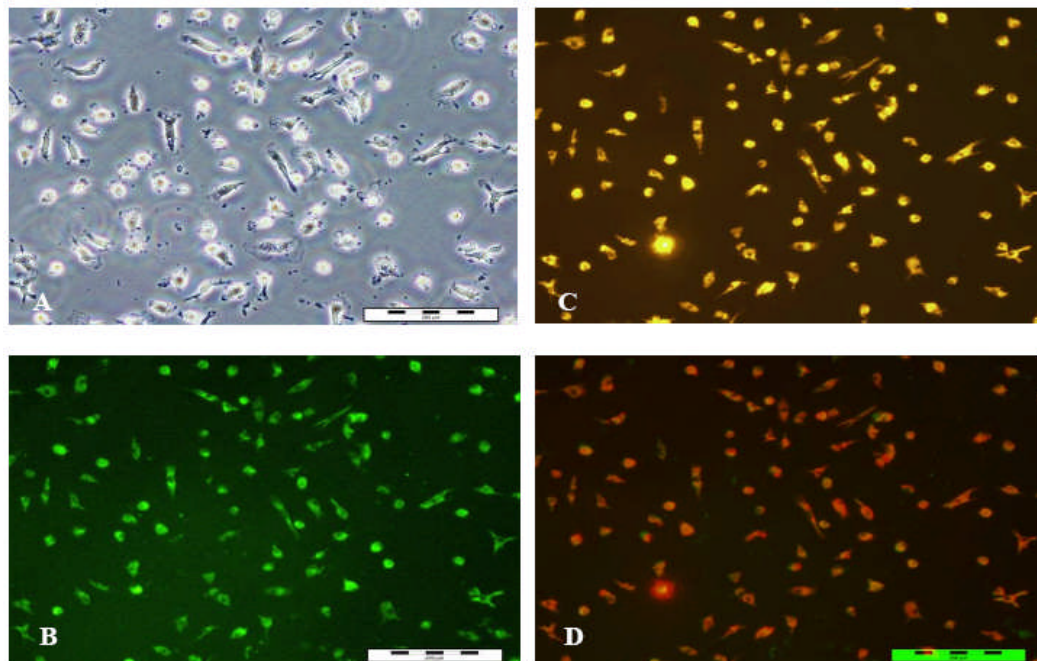# Results

CAC Characterisation

Figure 1. Fluorescently labelled CACs on a fibronectin coated tissue culture plastic **A.** Bright field microscopy of CACs, **B**. UAE-lectin stained CACs**, C.** ac-LDL stained CACs and **D.** Overlay of acetylated LDL stained and UAE-lectin stained CACs on tissue fibronectin-coated culture plastic. Cells co-staining for LDL and lectin are CACs.

CACs were characterized by uptake of acLDL and lectin. Figure 1 represents gross pictures of CACs on fibronectin coated tissue culture plastic. Dual stained cells are evident in figure 1D. There was no difference observed in metabolic activity of diabetic and non-diabetic human CACs.

Matrigel Tubule Formation Assay

Figure 2A is a representative image demonstrating increased tubule formation with HUVECs on matrigel which have been exposed to healthy CAC conditioned media Figure 2B demonstrates reduced tubule formation of HUVECs on matrigel which have been exposed to diabetic CAC conditioned media. There is a statistically significant difference in tubule formation of

HUVECs on matrigel with non-diabetic CACs having increased tubule formation. This experiment was performed in triplicate. (Figure 3)
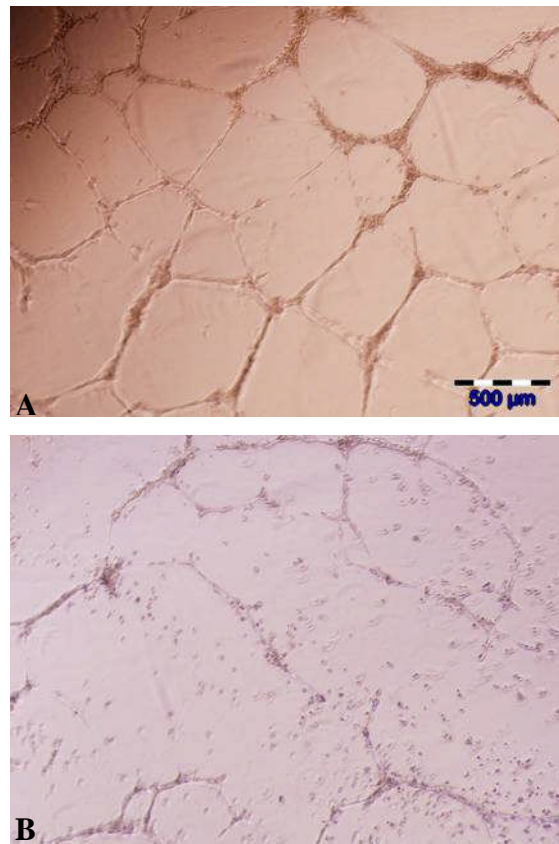

Figure 2. Representative Images (4X magnification) of matrigel tubule formation assay. **A** depicts the effect of conditioned media from non diabetic human CACs on tubule formation. **B** depicts the effect of conditioned media from diabetic CACs on tubule formation.
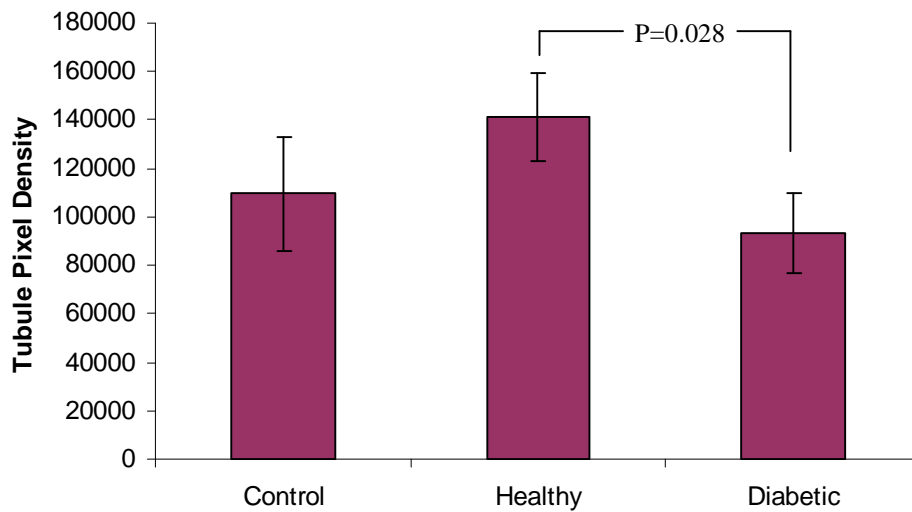
Figure 3. Matrigel assay of conditioned media from non-diabetic healthy CACs, diabetic CACs and media control. P value calculated using 2-sample t-test.

Angiogenesis-related protein production from CAC secretome

The angiogenesis-related protein assessment is represented in figure 4. For the 6 people with diabetes and the 6 people without diabetes, there were 6 angiogenesis-related proteins isolated and quantified from conditioned media experiments. These are detailed in table 1. There was no significant difference between the mean densitometry values of the proteins investigated between diabetic and non-diabetic CAC conditioned media. Other proteins identified included PEDF (Pigment epithelium-derived factor), Il-8 (Interleukin-8), Leptin, TSP-1 (Thrombospondin-1) These were isolated in 2 patients in addition to the other five proteins.
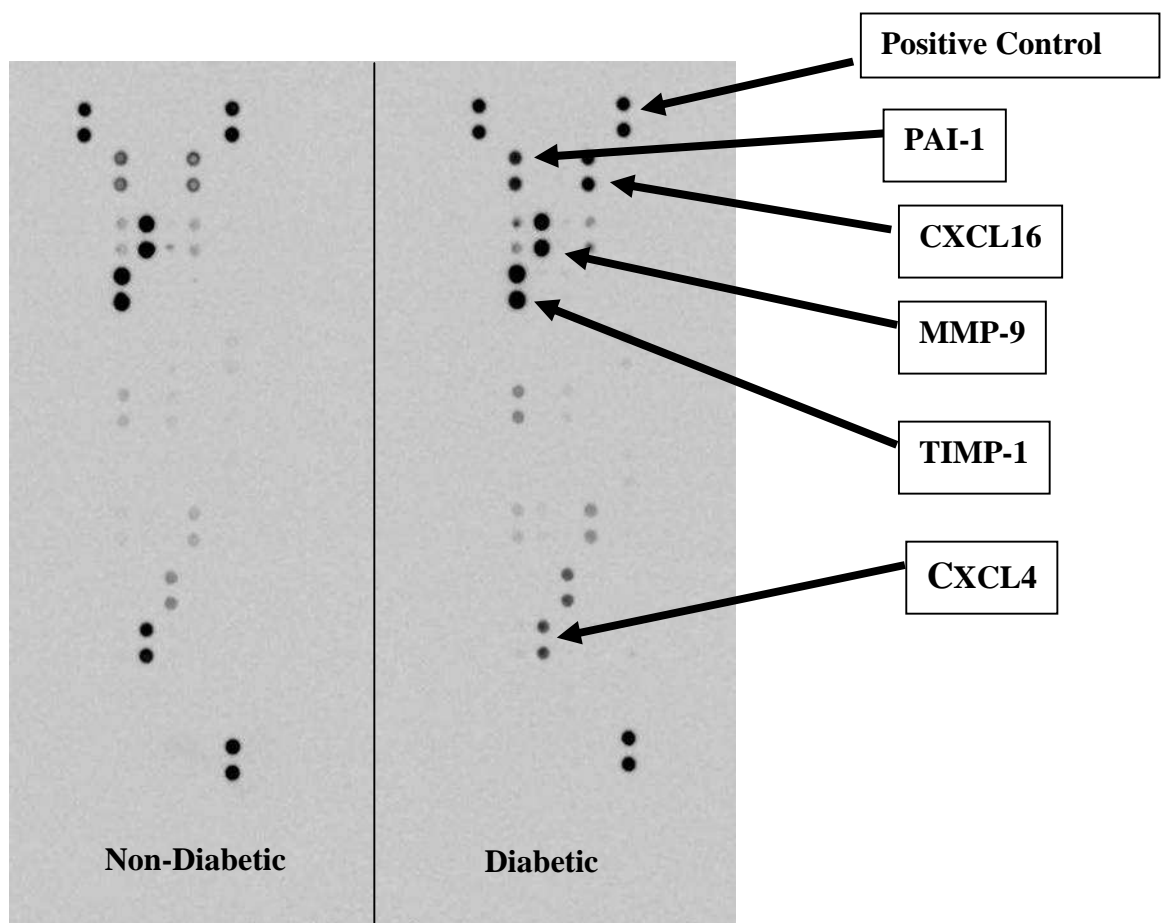
Figure 4. Angioarray data of diabetic and non-diabetic healthy CAC conditioned media.

Angiogenesis Protein	Arbitrary Densitometry Value (Pixels) ± SD		P Value
	Diabetic	Non-Diabetic	
MMP-9	1.609 ± 0.503	1.270 ± 0.796	0.399
TIMP-1	1.803 ± 0.486	1.621 ± 0.531	0.548
MMP-9/TIMP-1	1.139 ± 0.149	1.895 ± 1.646	0.276
CXCL 16	0.776 ± 0.267	0.619 ± 0.443	0.289
CXCL 4	0.57 ± 0.1083	0.655 ± 0.077	0.363
PAI-1	1.017 ± 0.454	0.939 ± 0.287	0.727

Table 1. Densitometry values for angiogenesis-related proteins from CAC conditioned media. n=6 for Diabetic and Non-Diabetic CAC conditioned media. Comparison of means analysed using 2-sample t -test.

Discussion

EPCs are thought to release multiple synergistic, therapeutic angiogenic factors which may explain the potent neovascularisation observed in animal models after EPC transplantation.[8] Previous research into endothelial progenitor cells has revealed that these cells produce proteins which are intimately involved in wound healing and angiogenesis. The interpretation of this research is complicated by the fact that different EPC subtypes were investigated.[8-10] Patients with diabetes mellitus have impaired angiogenesis which may be due at least in part to dysfunctional EPCs. In this chapter we sought to determine if diabetic EPCs are dysfunctional and if this is related to abnormalities in the secretion of angiogenic factors.

The angiogenic potential of cells is widely assessed using the matrigel assay.[6] It provides in vitro data comparing different treatments and permits decision making on which treatments are superior to translate to an in vivo model. In this chapter we demonstrate that human diabetic CAC conditioned media demonstrated decreased tubule formation using the matrigel assay when compared to non-diabetic human CAC conditioned media.

A pilot human study was performed to analyse the secretion of angiogenic factors by analysing CAC conditioned media from diabetic and non-diabetic subjects. The secretome was analysed using chemi-array technology as described above. Although a full proteomic analysis was not carried out, the proteins analysed were targeted as they are known to influence angiogenesis. A more detailed secretome analysis may be performed using mass spectrometry. This has been done previously with EPCs but not for people with diabetes.[11] The experiments were performed six times in non-diabetic and diabetic CACs. The subjects were age and sex matched and inclusion and exclusion criteria were strictly adhered to. CXCL-4, CXCL-16, PAI-1, MMP-9 and TIMP-1 were consistently isolated from both diabetic and non-diabetic CACs. There was no statistical difference observed in these proteins between diabetic and non-diabetic CAC conditioned media. It is not possible to draw conclusions with the other proteins identified including PEDF, Il-8, Leptin, TSP-1 as these were isolated only in 2 patients. Vascular Endothelial Growth Factor was not isolated from the conditioned media of diabetic and non -diabetic CAC conditioned media.

In summary in this study we demonstrate that CACs isolated from patients with diabetes mellitus has impaired ability to support tubulogenesis in an in vitro matrigel assay. However this observation does not appear to be related to differences in the secretion of angiogenic factors identified in the array used in the study. Limitations of the study include the limited number of patients studied.

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Supplementary Information for clinical trial

Invitation to take part in diabetes study investigating endothelial progenitor cells

Dear Patient

We would like you invite you to take part in a research project being carried out in the Diabetes Centre in the University Hospital Galway. Your participation would be very much appreciated. Please note:

- **All assessments in this study are free of charge**
- **You are under no obligation to take part and if you prefer not to participate, after reading the information about the study, we will accept your decision without question.**

This research study is being carried out by health professional from the Diabetes Centre and the regenerative medicine institute, national centre for bioengineering and science, National University of Ireland, Galway. The aim of the study is to investigate cells from blood. The cells are called endothelial progenitor cells and are important in new blood vessel formation. They are decreased in people with diabetes. We want to analyse these cells form your blood and find out what chemicals these cells produce. We want to analyse 100 millilitres of blood on two occasions.

1. At the beginning of the research study, and again at the end of the study which is predicted to be between 2 to 6 months. During that time we will provide advice and education on how to reduce your HbA1c, through adjustment of insulin doses, regular phone contact and monitoring of blood sugar glucose.

2. At the end of the study period we will take a second blood test and analyse the blood for endothelial progenitor cells and what they produce.

If you do decide to participate, you will be asked to attend for a 30 minute appointment which will involve discussion and assessment of your suitability to take part in the study.

I am enclosing a detailed information sheet about this research project and ask you to please read through this. <u>A member of the research team will contact you</u> by telephone and offer you an appointment. However, if you have any questions in the meantime, you can contact the diabetes centre on 091 542524.

Kind Regards,

_____

Dr. Aonghus O'Loughlin, Diabetes Centre, University Hospital Galway

Information Leaflet

The Effects of the control of diabetes on Endothelial Progenitor Cells

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A study is being carried out at the diabetes centre University college hospital, Galway and it is determining the effect of diabetes on endothelial progenitor cells. These are cells in the blood which are important in new blood vessel formation. In order to study these cells we need to investigate blood from people with diabetes. Below is an explanation of the study.

Ischaemia:

Patients with many diseases including diabetes mellitus, hypercholesterolaemia and renal failure, are prone to developing blockages in their blood vessels resulting in problems with the circulation of blood around the body. The muscles and organs supplied by these blood vessels have a reduced oxygen supply as a result of these blocked vessels leading to pain and eventual organ dysfunction. This condition is termed ischaemia. The main target organs include the heart, brain, kidney and the muscles of the legs.

Vasculogenesis:

Vasculogenesis is the term given to the growth of new blood vessels in the body to supply the ischaemic organs. It is particularly important in situations where the blockages in the blood vessels cannot be by-passed by surgical or angioplasty techniques.

Endothelial Progenitor Cells:

Endothelial progenitor cells (EPC's) are cells found in the blood stream that originate in the bone marrow. In recent years EPCs have been shown to incorporate into new blood vessels as they develop and thus play an important role in vasculogenesis. Studies are currently underway to examine the role of these cells in the growth of new vessels.

In this study, we want to investigate EPCs from people with diabetes. We would like to examine the effect of the blood sugar control on EPC number, proliferation and their ability to incorporate into new vessels in people with diabetes mellitus. We hope to learn more about these cells and their behaviour in diabetes so that further research may be able to improve new blood vessel growth and thus possibly help to develop future therapies for the treatment of ischaemic conditions and the complications of diabetes.

We need 100mls of blood from people with diabetes in order to isolate EPC's and study their function and also what these cells produce. We are looking for people with diabetes and no other medical conditions, who are not taking any medication except insulin, and do not smoke. We are looking for people without any complications of diabetes.

Participation in this study is entirely voluntary. If you do not wish to donate blood sample, there will be no record of this, and we will accept your decision without question, and you're further treatment will not be affected. Isolated cells may be stored for future research. There will be no identifying features associated with blood samples e.g. name or date of birth. The blood samples will be labelled with the persons age and sex e.g. 25 year old female. The research will be carried out by health professionals from the diabetes centre.

The researchers are Dr. Aonghus O'Loughlin and Professor Timothy O'Brien from University College Hospital, Galway. If you have and additional questions, they can be contacted through the Department of Medicine. Telephone 091-544206, 087 1262577.

CONSENT FORM

I..

of...

....

consent and agree to donate 100mls of blood drawn from .... to the Regenerative Medicine Institute at NUI Galway, for the purpose of cell isolation and research into the growth of new blood vessels in diseased states. The results of the information obtained from this blood will be kept confidential. Isolated cells may be stored for future research.

I have been given the patient information leaflet outlining the background of the study. I understand the procedure as explained to me by Dr./Mr. ....

Signature of Patient / Parent / Guardian* _____

Date   _____

Chapter 5

Topical Administration of Allogeneic Mesenchymal Stem Cells Seeded in a Collagen Scaffold Augments Wound Healing and Increases Angiogenesis in the Diabetic Ulcer

Diabetes mellitus is reaching epidemic proportions worldwide. Diabetic foot ulceration is the most frequent reason for hospitalisation and non healing ulceration may progress to amputation in spite of current standards of care. A central pathological factor in non-healing diabetic ulcers is an impaired blood supply. Topically applied mesenchymal stem cells (MSCs) provide a novel treatment to augment diabetic wound healing. Allogeneic non-diabetic bone-marrow derived mesenchymal stem cells were seeded in a collagen scaffold. The cells were applied topically to a full thickness cutaneous wound in the alloxan-induced diabetic rabbit ear ulcer model in a dose escalation fashion. The groups included: untreated wounds, collagen scaffold alone, collagen seeded with 50,000 MSCs, collagen seeded with 100,000 MSCs and collagen seeded with 1,000,000 MSCs. Percentage wound closure at 1 week was assessed using wound tracings. Angiogenesis was assessed using stereology, Inflammation was assessed by determining inflammatory cell infiltrate in the wounds. 1,000,000 MSCs demonstrated increased percentage wound closure when compared to control. Collagen and MSC seeded scaffolds demonstrated increased blood vessel density and decreased radial diffusion distance when compared to control. There was no significant difference in inflammatory cell infiltrate between groups. Allogeneic non-diabetic MSCs seeded in a collagen scaffold demonstrated efficacy in increasing cutaneous wound healing in a pre-clinical model. Collagen and collagen seeded with MSCs treatments result in increased angiogenesis when compared to untreated wound but an improvement in wound healing was only observed at the highest cell dose. This cell-based therapy provides for an increased wound closure and increased angiogenesis which is a central patho-physiological deficit in the non-healing diabetic foot ulcer.

Introduction

Non-healing diabetic foot ulceration poses a major burden on individual patients' health and healthcare budgets. Foot ulceration will affect 15-25% of people suffering from diabetes throughout their lives.[1] Diabetes-related lower extremity amputation arises from pre-existing ulceration in 85% of cases.[2] The high rate of progression from ulceration to amputation occurs despite standard care protocols. A central pathological factor in the treatment of non-healing diabetic ulcers is impaired angiogenesis in the wound.

There is a critical clinical need to develop novel treatments to improve healing of diabetic foot ulcers. Mesenchymal stem cells provide a novel therapeutic treatment. MSCs are beneficial in diabetic wound healing.[3] The mechanisms of wound healing benefit include paracrine secretion of growth factors and chemokines requisite for wound healing, and the differentiation into keratinocytes and endothelial cells required for wound healing and angiogenesis. They can be delivered in an allogeneic fashion, and possess immunosuppressant and immunomodulatory effects.[4]

To date, there have been encouraging preclinical results in animals models of diabetic wound healing. 10 humans have received autologous mesenchymal stem cells with augmented wound healing observed. There have been no studies using allogeneic human MSC transplantation in the setting of diabetic cutaneous ulceration. There is one report of dose effect with autologous MSCs seeded in a fibrin spray.[5] There exists a paucity of data on effective dosing strategies in the literature. Current regulatory requirements required that information on dose of cells be provided to ensure safety.

The use of biomaterials in vivo may ensure sustained viability and functionality of cells.[6] Collagen supports angiogenesis.[7] A collagen biomaterial allows targeted delivery and positioning of high numbers of cells at the wound site. We hypothesised that topical application of a collagen scaffold seeded with allogeneic non-diabetic bone marrow derived MSCs supports angiogenesis and augments cutaneous wound closure in a diabetic animal model of cutaneous

wound healing. We investigated the therapeutic effect of collagen seeded MSC therapy in a robust pre-clinical model using wound tracings and stereology. This technique is a scientifically robust validated strategy to assess in vivo tissue responses to bioengineered living tissue constructs.

Materials and Methods

MSC Culture and Characterisation

Animal experiments were carried out under a licence from the Department of Health, Ireland and the National University of Ireland Galway ethical Committee. Allogeneic rabbit non-diabetic bone marrow derived mesenchymal stem cells were donated from REMEDI laboratory staff. The cells were previously isolated form male New Zealand Whit Rabbits. These MSCs were characterised by differentiation assays to confirm cell differentiation into chondrogenic, osteogenic and adipogenic lineages.[8] 200,000 MSCs aliquots were frozen in liquid nitrogen at passage 3 and these cells were used for future experiments.

Collagen scaffold and Cell seeding

Type 1 bovine collagen solution was isolated and purified as described previously.[9] A collagen sponge was created by pipetting 500 µl of 3% (weight) type 1 bovine atelocollagen solution into 48 well tissue culture plates (Sarstedt Ltd., Wexford, Ireland). This was then lyophylised using a VirTis freeze-dryer (Suffolk, U.K) The collagen sponge was prepared by washing once with HANKs balanced salt solution (sigma), 3 washes with 70% ethanol, 2 washes of sterile water (Sigma), and 2 washes of EBM-2 (Lonza) media. After the washing steps the collagen scaffold was transferred to one well of a 48 well cell culture plate (Sarstedt Ltd., Wexford, Ireland). The frozen aliquots of MSCs were plated in a T75 tissue culture flask. (Nunc) After 4 days, confluent MSCs were trypsinised and seeded by injecting cells in 1000 µl of alpha MEM supplemented media using an insulin syringe. (Becton, Dickinson and Company) Cells were placed in an incubator for 16 hours in 37°C and 5% CO_2. Prior to application to the wound,

the cell scaffold was washed three times with serum free media and twice with phosphate buffered saline.

Metabolic Activity and Fluorescent Labelling of MSCs

The metabolic activity of cells was assessed using Alamar blue (rezasurin) (Invitrogen). 24 hours after seeding, the cells were washed once in Hanks balanced salt solution (Sigma) and incubated for 3 hours in 10% alamar blue. This was performed at 24, 96, 144 and 366 hours. This was performed for 50,000 and 1,000,000 rabbit MSCs seeded on a collagen scaffold. The absorbance of each sample was measured in a 96-well plate at wavelengths of 550 and 595nm using a microplate reader. The percentage of reduced Alamar blue was determined as previously described.[10] In one animal experiment MSCs were labeled using PHK-26 (Sigma) according to manufacturers instructions and the cells were imaged using a fluorescent microscope (Olympus).

Scanning Electron Microscope

Scaffolds and scaffolds seeded with MSCs were rinsed 0.1M Phosphate buffer, pH 7.2 and fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer for 2hrs at room The samples were dehydrated with ethanol and then placed in hexamethyldisilazane for 30 minutes. The samples were then gold-coated and analysed using a scanning electron microscope (Hitachi S-4700)

In-vivo Model

Nine New Zealand white rabbits (3-3.5Kg) were used in the study. The protocol was approved by the ethics committee of the National University of Ireland, Galway and the study conducted under a license granted by the department of Health and Children Dublin, Ireland. Rabbits were housed in individual cages and with a 12h light/dark cycle and controlled temperature and humidity. Rabbits were fed a standard chow and water *ad libitum*.

Induction of hyperglycaemia

Rabbits were sedated with intramuscular injection of ketamine, xylazine and acepromazine. Hair was shaved off the back of the ears. Alloxan (150 mg/Kg) (Sigma-Aldrich) was made up in 30 mL of saline and administered via an ear vein using an intravenous cannula at a rate of 1.5 mL/min. After treatment water containing glucose was provided for 24 hours in addition to provision of molasses to the animals' front feet to prevent hypoglycaemia. Serum blood glucose was checked daily using Accucheck advantage strips (Roche). Insulin therapy was administered if the animal lost weight and had high glucose readings using insulin glargine (Sanofi-Aventis). High glucose readings were indicated on the glucometer as 'hi' and signified serum glucose of greater than 33 mmol/L.

Surgical Procedure

After 5 weeks of hyperglycaemia, rabbits were anaesthetised using intramuscular injection of 0.1 mL/Kg xylazine and 0.12 mL of ketamine which is half dose analgesia. Sterile disposable 6 mm punch biopsies were used to create 3 wounds on one ear and 2 wounds on the other ear. The wounds were created and dermis exposed to bare cartilage. Each wound was treated with one of five randomized treatment groups: 1. no treatment, 2. collagen scaffold alone, 3. collagen scaffold seeded with 50,000 MSCs, 4. collagen scaffold seeded with 100,000 MSCs and 5. collagen scaffold seeded with 1,000,000 MSCs. The MSC-scaffold treatment was applied with the superior surface of the construct, which contained the majority of cells being applied to the base of the wound. The wounds were covered with a polyurethane dressing (Opsite) and the ear was stitched and covered with adhesive dressing (Operfix), until day 7 (n=9). The animal received 5 mg/Kg enfloxacin antibiotic (Baytril, Bayer USA) and opiate analgesia post-operatively. At 7 days rabbits were euthanized with intravenous sodium pentobarbital (2 mL).

Wound Closure

Wound closure was assessed as previously described.[11] The wound was traced on the day of sacrifice. A fresh wound was made on the day of sacrifice and the percentage wound area reduction over 1 week was calculated using formula A.

Histology

The wounds were cut across the midline and fixed in 10% formalin for 24 hours. The tissue was processed using a tissue processor (ASP300 Meyer) and embedded in paraffin. 5 micrometer sections were taken when the tissue was reached. 6 sections were cut using a microtome every 150 micrometers into the wound for analysis. Three sections were placed on one slide. Sections were stained with 1. Haematoxylin and eosin, and 2. Masson's trichome using standard protocols.

Wound Volume and Epithelialisation

Wound Volume was calculated by multiplying the average wound thickness by the area of the wound tracing one week after wounding. Six measurements were taken from the cartilage to the wound surface and measured using Cell B software, and the average thickness calculated.

Epithelialisation was assessed by measuring the horizontal distance between the two wound edges (Figure 1 (A)). The wound edge was determined by a change in thickness of the epithelium, a lack of sebaceous glands, hair follicles and skin appendages. The length of the newly formed epithelium was measured (figure 1 (B+C)) and a percentage was obtained. This was performed on the first section analyzed from the centre of the wound.
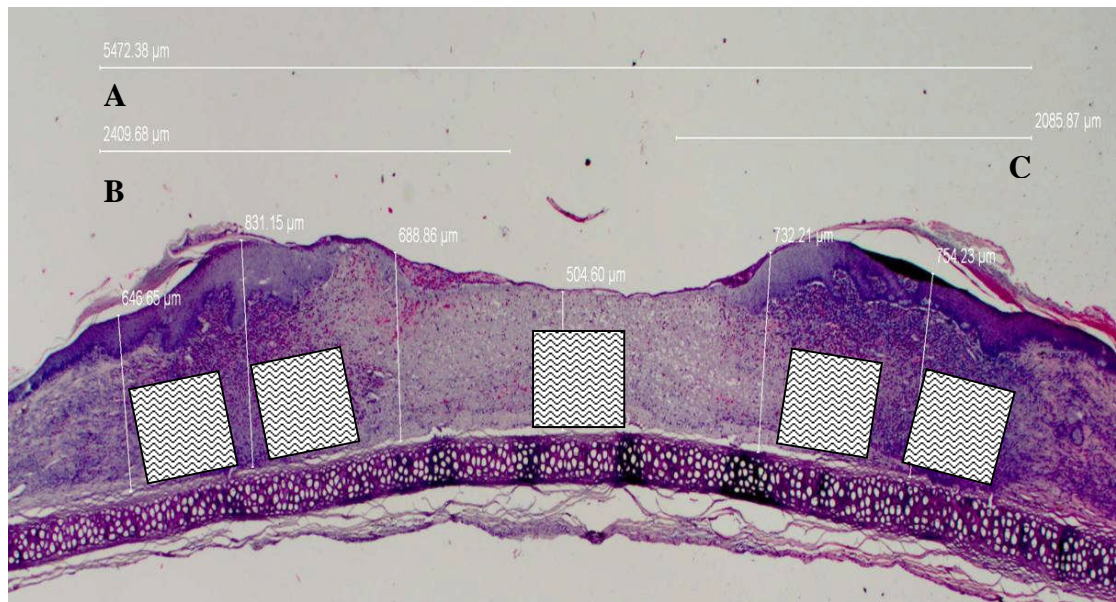
Figure 1. Cross sectional image of Wound stained with haematoxylin and Eosin. Wound thickness is calculated from 6 measurements across wound. The boxes across wound are where the images are taken for stereological assessment. Epithelialisation is calculated from distances A, B and C.

Stereology

Stereology is a means of assessing tissue responses to tissue constructs. It allows assessment of angiogenesis in vascular beds.[12] A series of cycloid lines were placed on the histology sections using image pro plus software. (figure 2) In order to ensure the areas of the wound had the same chance of being selected, selection of the fields was done in a random manner. Five fields of view were obtained across the wound bed from one edge of the wound to the other edge.[11] The fields were captured at 20X magnification.

The parameters assessed were surface density of blood vessels, length density of blood vessels and radial diffusion distance between capillaries. Surface density (S_V) represents the amount of surface area (S_A) contained in a reference volume (V). The surface area of a capillary represents the area available for gaseous transport to surrounding tissue. The higher the surface area of a capillary network, the higher the probability that the surface will intersect parallel lines placed on the image. Length density is a measurement of the length of blood vessel per unit volume of tissue (Lv), which is based on the principle that the

132

longer and more convoluted a vessel, the greater the number of occasions its profile intersects a plane.[11,12]

Length density and surface density of blood vessels were analysed with and without multiplying by the wound volume. The surface density of blood vessels was calculated using Formula C and the length of test line was 2483nm. The surface area of blood vessels was then calculated by multiplying the surface density by wound volume. To calculate the length density of blood vessels, a series of cycloid lines measuring 2649nm in length were rotated 90 degrees and placed on the histological section. The length density of blood vessels was calculated using Formula D. The total length of blood vessels in the wound was calculated by multiplying length density by wound volume. The radial diffusion distance was calculated using Formula E. This allows for the measurement of the distance between blood vessels, and is an indicator of the efficiency of a capillary network. The smaller the distance between blood vessels, the shorter distance required for nutrients to diffuse into surrounding tissues. Blood vessel diameter was calculated using Formula E.[13]

Volume Fractions

The volume fraction of a feature within a particular reference space can be described as the proportion of space that the feature occupies in a unit volume.[11] Inflammatory cells were counted and included lymphocytes and neutrophils. This was counted using a 192 point grid using Image Pro Plus software (Media Cybernetics). (Figure 3) Neutrophils were identified as small dense circular multi-lobed cells and lymphocytes as small round dense cells with large nuclei.

Formulae for Stereology [11,13]

A. Length Density

$$L_V = \frac{(2 \times I_L)}{T_S}$$

B. Radial Diffusion Distance

$$R_{diff} = \frac{1}{\sqrt{\pi \times L_v}}.$$

C. Surface Density

$$S_V = 2 \times \frac{I}{L_T}$$

D. Blood Vessel Diameter

$$d = \frac{S_V}{L_V \cdot \pi}$$

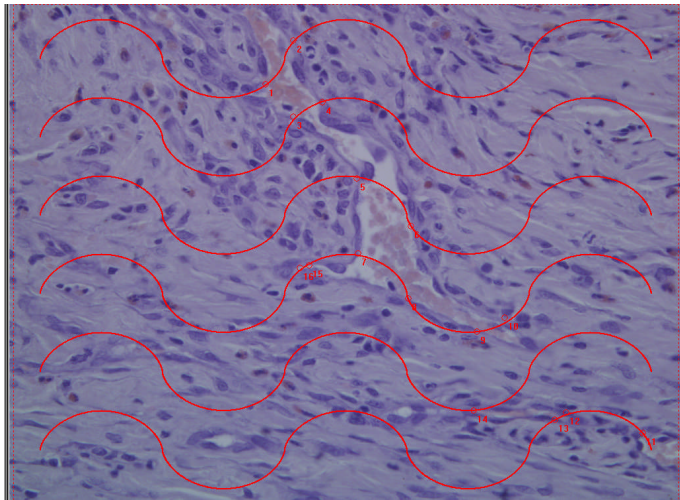**E. Inflammation Cell Fraction**

$$= \frac{P_P}{P_T}$$

Figure 2: Haemaotoxylin and Eosin stained section of wound at 7 days. A cycloid grid used for stereological analysis. (Intersections with blood vessels and gridlines are marked.)
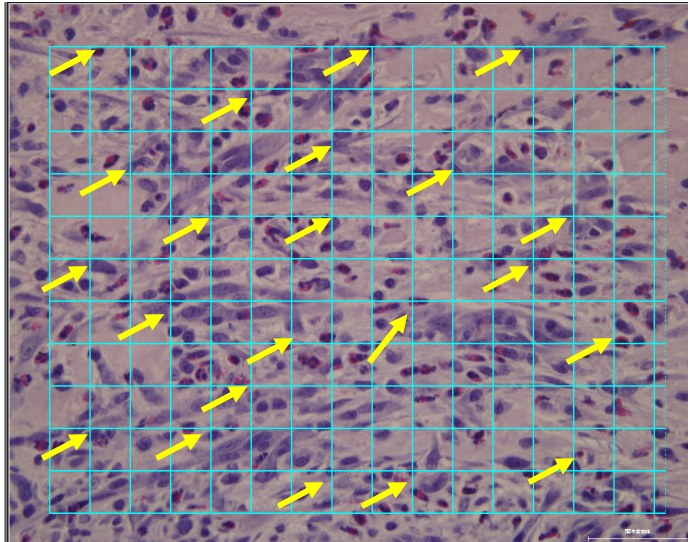
Figure 3. Haematoxylin and Eosin stained sections of normal wound at 7 days. Calculating the volume fraction of inflammatory cells using a 192 point grid. Arrow indicates examples of inflammatory cells on intersection of the grid. (Images taken at 20X Magnification.)

Statistics.

Analysis between groups was assessed using Analysis of Variance and post hoc analysis with Fisher's pairwise comparison. $P < 0.005$ was taken as significant. Minitab software was used.

Results

Animal Model.

The animals remained hyperglycaemic post alloxan infusion over the study time period (figure 4.). There was no mortality post alloxan treatment. Two animals required insulin administration after alloxan treatment.
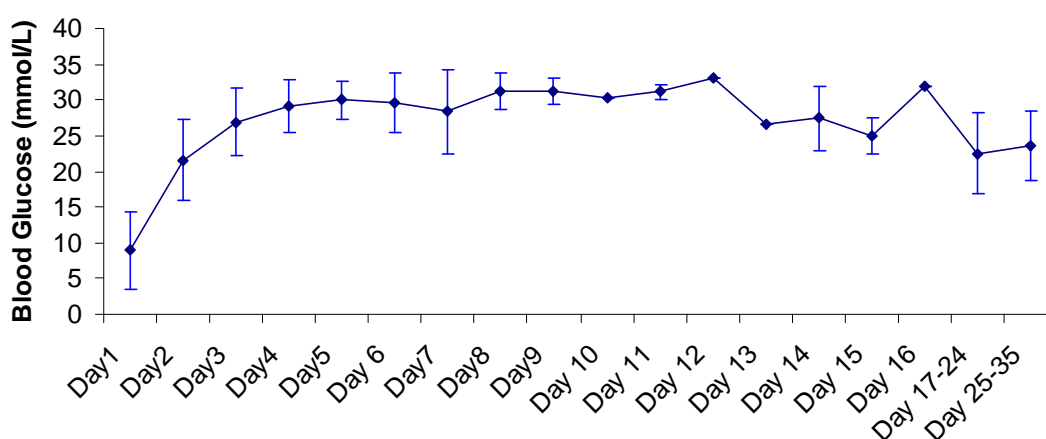

Figure 4. Blood Glucose Readings of rabbits post alloxan treatment. The serum glucose of animal remained > 20 mmol/L for 35 days post alloxan up until time of surgery and treatment application.

MSC Culture and Characterisation.

MSC were successfully isolated from non-diabetic rabbit bone marrow. Cells were cultured to passage 3 and frozen in liquid nitrogen in 200,000 doses. Images of passage 4 MSCs are represented in figure 5. MSCs on tissue culture plastic demonstrated spindle shaped morphology on becoming confluent. MSCs differentiated into chondrocytes, osteocytes and adipocytes. (Isolation of cells and differentition assays performed kindly by REMEDI staff) Cell surface immunophenytyping was not carried out due to the lack of rabbit specific antibodies available.

Metabolic Activity of Cells

Rabbit MSCs retained metabolic activity after seeding for 24 hours, up until 2 weeks in vitro as compared to media control. This was observed for rabbit MSCs seeded at an initial dose of 50,000 per collagen scaffold and 1,000,000 per collagen scaffold (Figure 6).
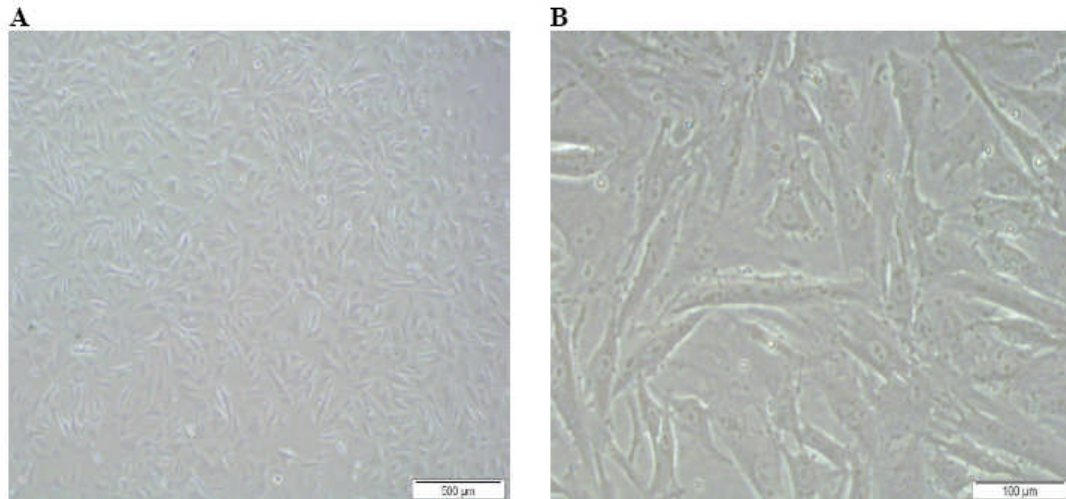

Figure 5. Gross pictures of passage 4 rabbit MSCs on tissues culture plastic. 4X magnification **(A),** and 20X magnification **(B)**.
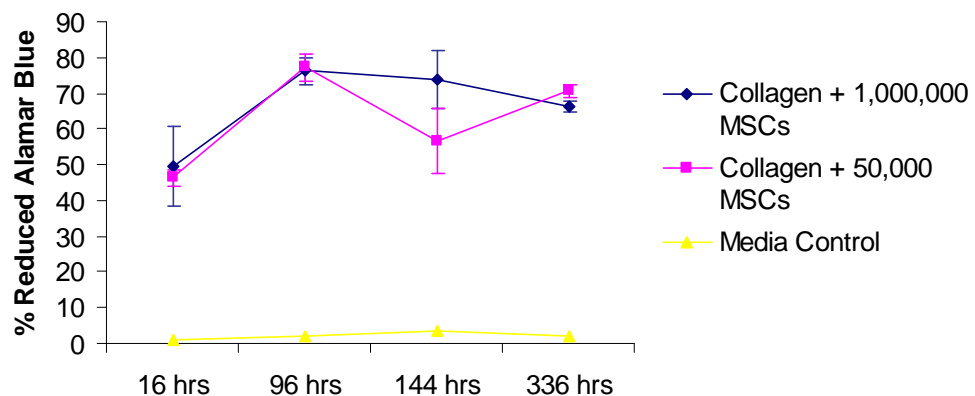

Figure 6. Time-course of Metabolic Activity of MSCs seeded on a collagen scaffold. Metabolic activity is assessed using Alamar Blue (resazurin) (Invitrogen). The assay was performed at 16 hours, 96 hours, 144 hours and 336 hours. The metabolic activity of rabbit MSCs seeded on a collagen scaffold is maintained for two weeks in vitro as compared to media control. This is evident for cells seeded at doses of 50,000 and 1,000,000 cells per scaffold.

Histology and Scanning Electron Microscopy

After immersion fixation, tissue processing and sectioning of the MSC-collagen constructs, haematoxylin and eosin and mason's trichome staining were performed. Cells were predominantly located on the superior border of the collagen scaffold. Figure 7 represents 1,000,000 rabbit MSCs seeded in a collagen scaffold and stained with Mason's Trichome (A, C and D), and haematoxylin and eosin (B).
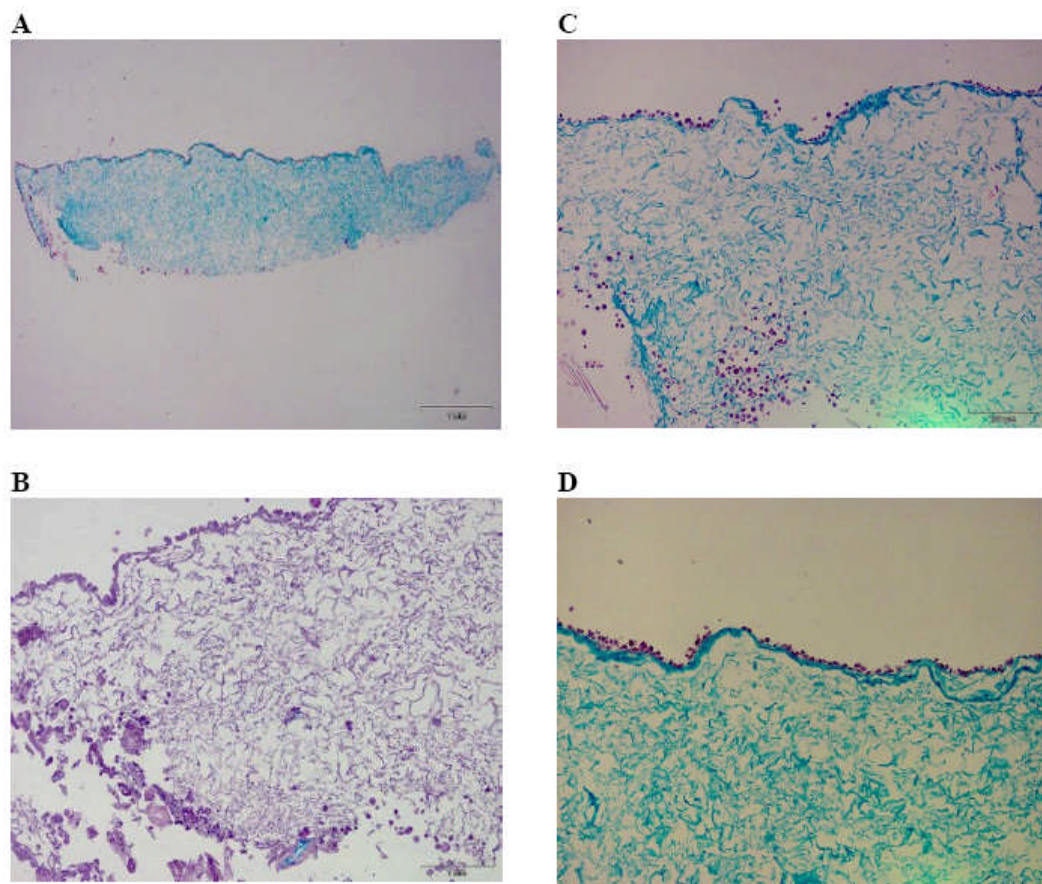

Figure 7. Histological Sections of collagen scaffold seeded with 1,000,000 rabbit MSCs. **A.** Masson's trichome stained section 2X magnification. **B.** Haemotoxylin and Eosin stained section 10X magnification. **C.** Masons Trichome stained section 10X magnification. **D.** Masson's trichome stained section 20X magnification. MSC are located predominantly on the upper surface of the collagen scaffold. This surface of the scaffold was applied to the surface of the wound. (Green = collagen, purple = MSCs)

Scanning electron microscopy images (figure 8, A-D) revealed densely populated MSCs with the collagen scaffolds seeded with 1,000,000 cells.
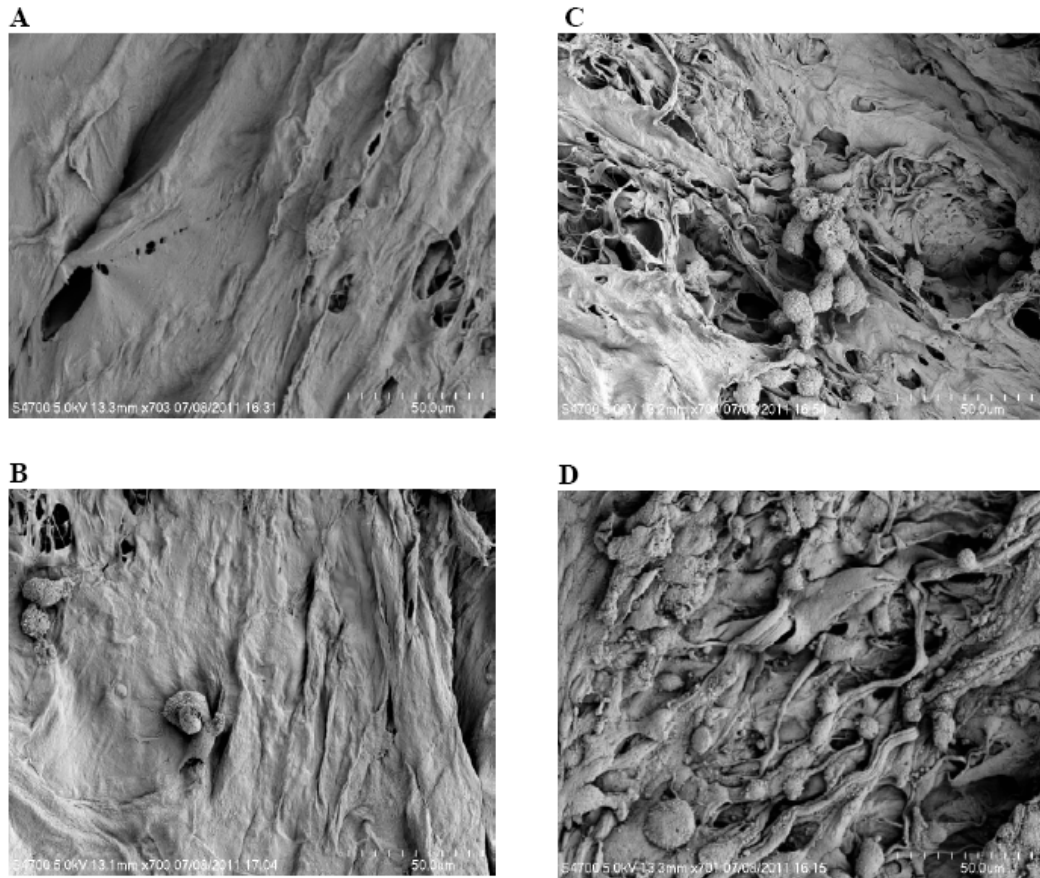

Figure 8. Scanning Electron Microscopy images of rabbit MSCs 24 hours after seeding on a collagen scaffold. **A**. Unseeded scaffold, **B.** Scaffold seeded with 50,000 MSCs, **C.** Scaffold seeded with 100,000 MSCs, **D.** Scaffold seeded with 1,000,000 MSCs. The cells were adherent to the scaffold. MSCs were confluent on the scaffold at a at a dose of 1,000,000

Cell Tracker.

Figure 9 demonstrates PKH-26 labelled rabbit MSCs in the wound 1 week post treatment. This provides evidence that the cells were located in the wound site at one week post treatment. The cells co-localised with DAPI stained cells. This reveals MSCs were present in the wound at 7 wounds and that the collagen scaffold was successful in mediating cell delivery to the wound.
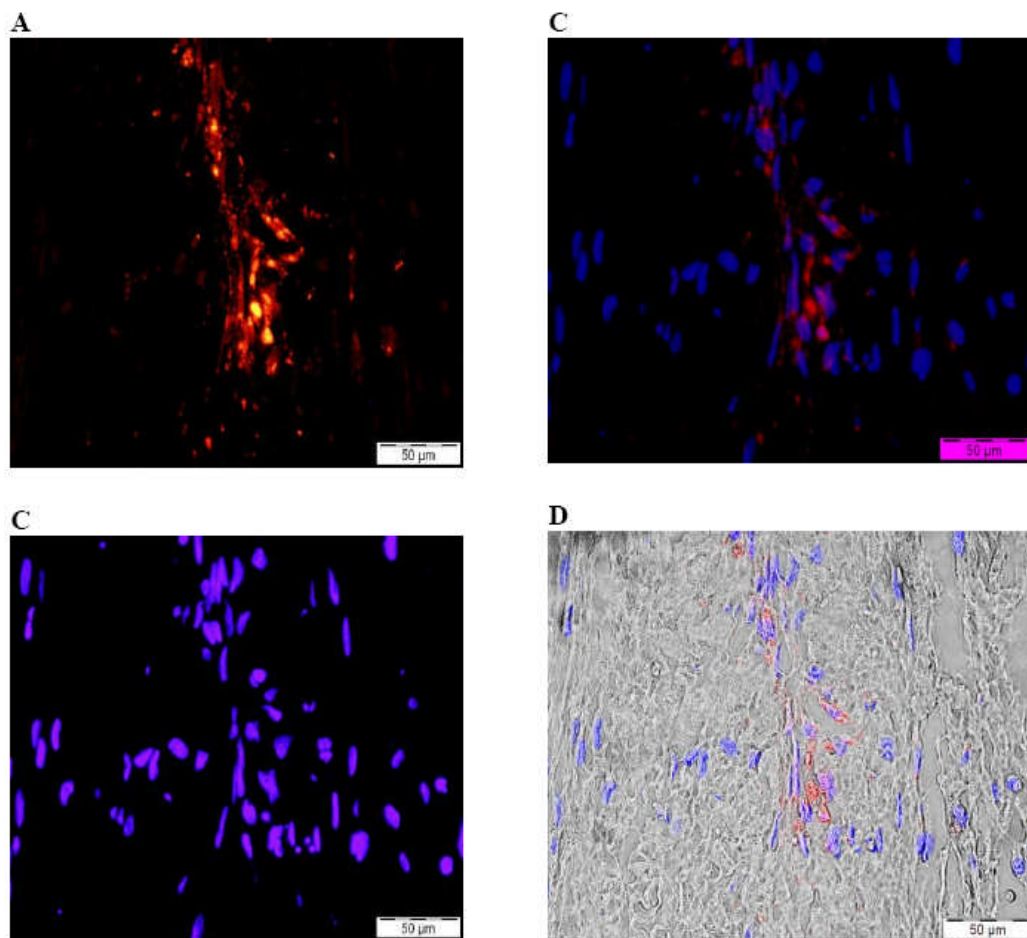

Figure 9. Fluorescently labelled MSCs in diabetic wound 1 week after treatment. (40X Magnification) **A.** PKH26 labelled MSCs (red). **B.** DAPI stained cells in wound. (blue) **C.** Overlay of Labelled MSCs and DAPI stained cells. **D.** Overlay bright-field image and labelled MSCs (red) and DAPI stained cells (blue.)

Histology

Figure 10 illustrates representative samples of Masons' Trichome stained histological sections of rabbit MSCs seeded in a collagen scaffold and delivered

to an ulcer in a diabetic animal. MSCs delivered in a collagen scaffold demonstrates increased healing in MSC treatment group as compared to untreated wound and wounds treated with collagen There is increased new granulation tissue in the wound and a more organized wound healing response (figure 10D-10F).
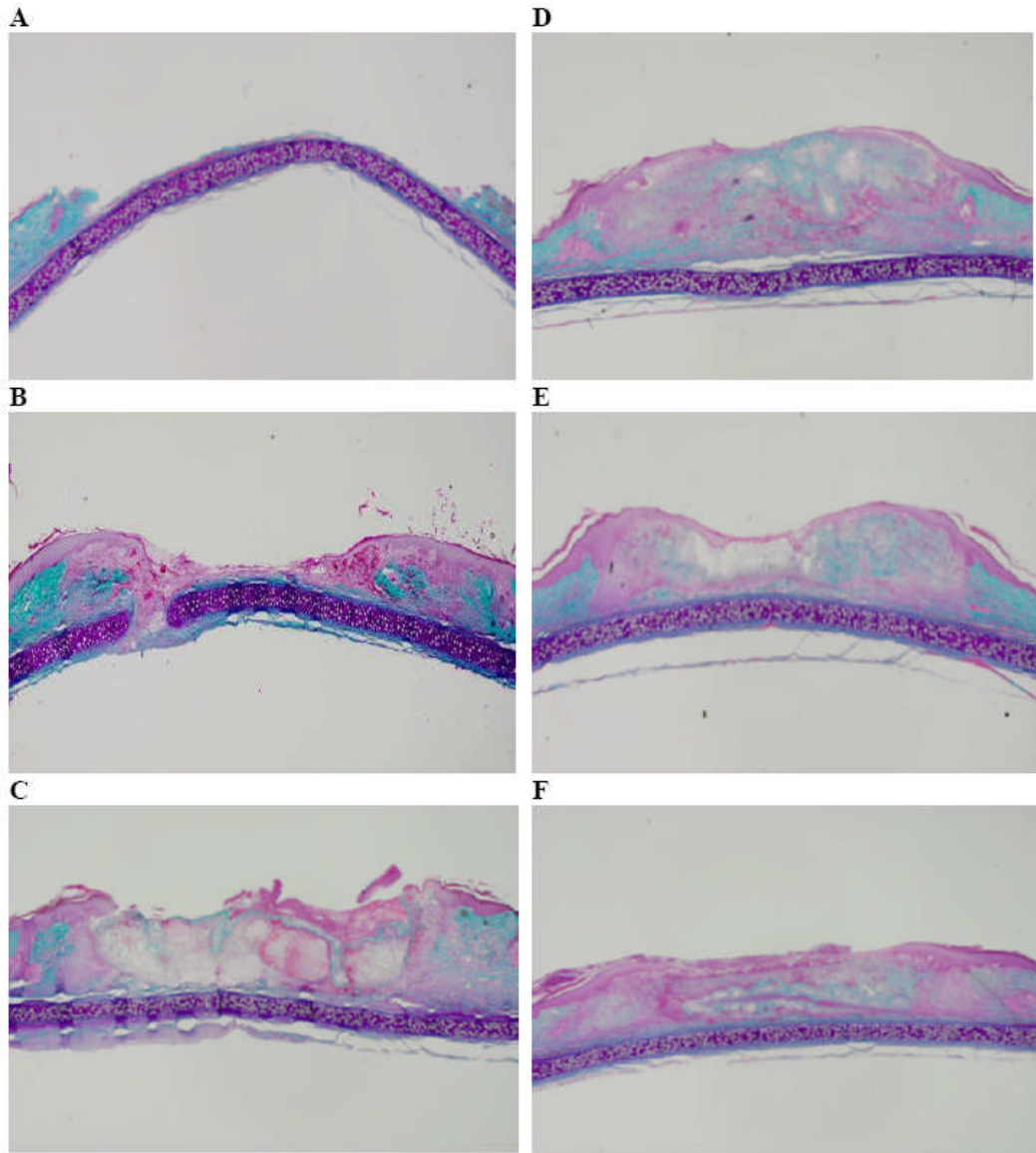

Figure 10. Mason's trichome stain of rabbit ear ulcer wounds. **A.** Fresh wound made on day of sacrifice. **B** Untreated wound after 1 week. **C.** Wounds treated with collagen after 1 week. **D.** Wounds treated with collagen + 50,000 MSCs after 1 week. **E.** Wounds treated with collagen + 100,000 MSCs after 1 week. **F.** Wound treated with collagen + 1.000,000 MSCs after 1 week. Green stain represents collagen. Pink stain represents cytoplasm and epithelium. Purple stain represents cartilage. Images taken at 2X magnification.

Percentage Wound Closure

1,000,000 rabbit MSCs seeded on a collagen scaffold demonstrate statistically increased percentage wound closure as compared to untreated wound. There was no statistical difference in percentage epithelialisation between groups 1 week after treatment.
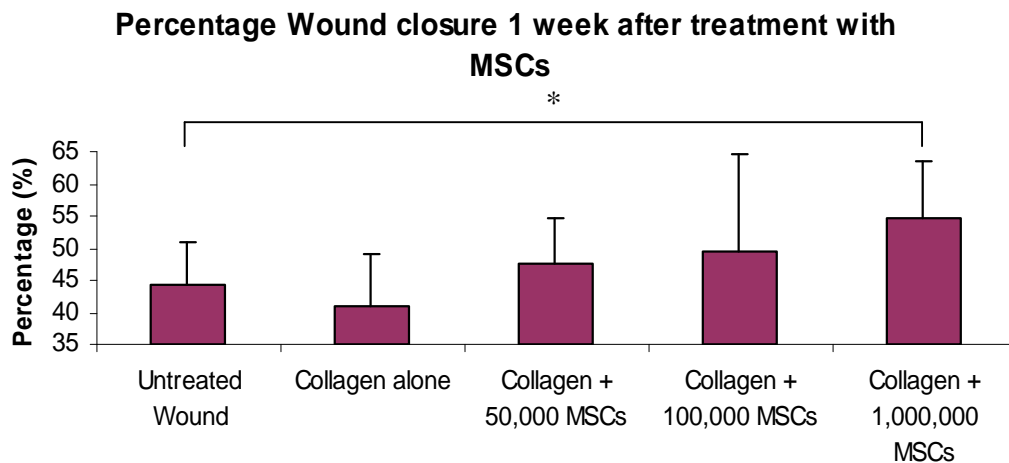

Percentage Wound closure 1 week after treatment with MSCs

Figure 11. Percentage wound closure of cutaneous ulcers one week after treatment with MSCs seeded in a collagen scaffold. Analysis between groups using ANOVA and Fishers Pairwise comparison. $*P<0.05$. Error bars = Standard Deviation.

Stereology

Wound volume is significantly less in wounds treated with collagen seeded with 50,000 and 100,000 MSCs compared to untreated wounds. There is no difference between the wound volume of 1,000,000 MSCs seeded in a collagen scaffold as compared to untreated wound and collagen treated wound (Table 1).

Stereological analysis (Table 1) demonstrates significantly increased total length of blood vessels in wounds treated with 1,000,000 MSCs seeded on a collagen scaffold as compared to untreated wound.

The surface density of blood vessels in wound treated with collagen and collagen seeded with MSCs is significantly increased as compared to control. This indicates a significantly increased area of blood vessels present in wound to

ensure a greater area of capillaries available for gaseous exchange. The length density of blood vessels is significantly increased in wounds treated with collagen and collagen seeded with MSCs when compared to untreated wounds. This indicates longer blood vessels in these wounds. The neovasculature in these wound demonstrate longer more convoluted wounds as compared to untreated wounds. This vasculature is more efficient than untreated wounds. In addition on adjusting the length density for wound volume, the total length of blood vessels in wounds treated with collagen seeded with 1,000,000 cells is significantly longer than control wounds. Increasing the dose to 1,000,000 MSCs demonstrates a more efficient neovasculature as compared to untreated wounds.

Blood vessels in collagen treated wounds and collagen seeded with MSCs demonstrate significantly reduced radial diffusion distance when compared to untreated wound. This occurs across all doses of MSCs. The distance for nutrients to travel from capillaries to tissue and cells is reduced and permits augmented tissue repair and regeneration. There was no statistical difference in blood vessel diameter between groups 1 week after treatment.

Inflammation can be assessed in tissues using stereology. Inflammatory cell infiltrate is increased in healing tissue. In addition inflammation may be increased in response to tissue engineered biological construct implantation. The use of stereology which quantifies neutrophil and lymphocyte infiltrate in tissue can assess inflammation in wounds one week after treatment. No significant difference was observed in infiltrate of inflammatory cell in any of the groups, i.e. untreated wound and wounds treated with either collagen alone or collagen seeded with MSCs.

Parameter	1 x10^6 MSCs + Collagen	100,000 MSCs + Collagen	50,000 MSCs + Collagen	Collagen	Untreated Wound
Wound Volume (mm^3)	23.44±6.6	20.48±6.17[#]	22.55±4.23[#]	24.39±5.4	29.27±9.04
Volume of Inflammatory Cells (mm^3)	3.6±1.9	3.55±1.6	3.465±0.94	3.08±1.13	2.418±1.02
Surface Density of blood vessels in wound (mm^{-1})	56.81±20.14[#]	53.25±9.79[#]	52.68±10.7[#]	46.24±11.21[#]	31.03±6.84
Surface Area of Blood Vessels (mm^2)	1393±781	1094±409	1196±345	1135±378	929.6±459
Length Density of Blood Vessels in Wound(mm^{-2})	11140± 3737[#]	11264± 2394[#]	10969± 2312[#]	9627±2711[#]	5425±1591
Total Length of blood vessels in wound (mm)	270731± 146549[#]	231894± 90588	250521± 80213	234213± 75625	162924± 90070
Radial Diffusion Distance (microns)	5.576±0.11[#]	5.412±0.68[#]	5.495±0.75[#]	5.891±0.782[#]	7.919±1.33
Vessel Diameter (microns)	1.626±0.19	1.52±0.13	1.55±0.2	1.54±0.19	1.90±0.42

Table 1. Stereological analysis of wounds in diabetic animals. (Analysed by ANOVA followed by Fishers pairwise comparison, n=9), [#]$P<0.05$ compared to untreated wound.

Discussion

Topical MSC therapy is an attractive new treatment for non-healing diabetic foot ulceration which may lead to amputation. The goal of therapy is to reduce the time to wound closure. A central pathological factor in diabetic foot ulceration is impaired angiogenesis. MSCs are known to promote angiogenesis in addition to improve cutaneous wound healing.[15]

It is known that cells injected directly into the body undergo cell death rapidly.[6] Biomaterials may support cell viability and thus enhance therapeutic efficacy. In addition, previous reports show that MSC treatment when injected around the wound augments wound healing and increases percentage wound closure but fails to increase angiogenesis at the wound site.[14] In this research, MSCs were injected intradermally around the cutaneous wounds in diabetic rats. The authors investigated the angiogenesis endpoints in histological sections using similar stereological methodology as used here. This was performed after the wound healed. MSCs augmented wound healing but this was not associated with increased angiogenesis. Collagen is a natural biomaterial which promotes sustained cellular viability and functionality in addition to maintaining the cells at the wound site and was used to deliver MSCs to the wound surface in this study.

We investigated the effect of topical delivery of allogeneic non-diabetic bone marrow derived mesenchymal stem cells delivered to a diabetic wound in an immuno-competent animal. The use of MSCs allowed allogeneic transplantation from a non diabetic donor, an approach which may have advantages over autologous cell transplantation in which disease-induced cell dysfunction may limit therapeutic efficacy. We have previously described impaired wound healing in the diabetic model used in the current study.[11] It closely resembles the human situation as the wound heals by granulation tissue formation and epithelialisation. The wound is a full thickness cutaneous ulcer and facilitates assessment of wound closure and new granulation tissue formation. It is a non contractile wound and allows assessment of angiogenesis and inflammation. Using stereological methodology, a comprehensive assessment of host tissue responses to cell seeded biomaterial constructs can be achieved.

Non-diabetic bone marrow derived MSCs remained viable after freezing in liquid nitrogen and differentiated into three mesodermal cells, adipocytes, chondrocytes and osteocytes. Flow cytometric analysis was not performed as there is a lack of antibodies commercially available for rabbits and this was not pursued. MSCs were successfully seeded in the scaffold. The MSC-scaffold treatment retains cellular viability in-vitro for up to 2 weeks. This provides evidence for the use of MSCs which are frozen in liquid nitrogen as an "off-the-shelf" product. Rabbit MSCs were located predominantly on the surface of the scaffold. Histological and scanning electron microscopy demonstrated rabbit MSCs on the scaffold. Fluorescently labelled MSCs were located in the wound 1 week after treatment.

In this dose escalation study of topical MSC therapy, 1,000,000 cells revealed increased percentage wound closure when compared to control. This endpoint is highly relevant clinically. It provides a non-invasive measurement of wound healing and increased percentage wound closure is associated with accelerated wound healing. Stereological analyses performed through the wound provide information on tissue responses to topical treatments. Multiple histological sections were taken throughout the wound. Increased angiogenesis is reported in all treatment groups as compared to controls but enhanced wound closure was only observed in the high dose group. Both surface density and length density of were significantly increased in wounds treated with collagen alone and collagen seeded with MSCs when compared to untreated wounds after one week. In addition the radial diffusion distance was significantly less in wounds treated with collagen alone and collagen seeded with MSCs when compared to untreated wounds.   The radial diffusion distance is a measure of the efficiency of a capillary network. The total length of blood vessels in the wound is significantly greater in wounds treated with collagen seeded with 1,000,000 MSCs as compared to other wounds.   This increased blood vessel length suggests that at increasing doses of MSCs, to 1,000,000 in the case of this study that there is a more efficient blood vessel network, not seen with lower doses of MSCs.

The stereological analysis across groups revealed no statistical difference in inflammatory cell infiltrate between treatment groups and untreated wound. This is important to ensure that the tissue engineered construct does not illicit an immune

response due to the allogeneic nature of the MSCs and the xenogeneic bovine collagen scaffold.

These data provide evidence of the wound healing benefit associated with wounds treated with collagen and collagen seeded with MSCs. Collagen seeded with 1,000,000 MSCs results in a significantly increased percentage wound closure and a superior vascular supply when compared to untreated wound at one week. This is the first extensive analysis of MSCs delivered to a wound using a collagen scaffold. It confirms the wound healing benefit of MSCs occurs with increased angiogenesis as reported in previous studies and for the first time assesses the optimal dose and the use of a collagen scaffold for cell delivery.[15] This research suggests a treatment dose of 1,000,000 MSCs applied to full thickness circular ulcer resulted in an increased percentage wound closure and increased total length of blood vessels in wounds.

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Chapter 6

Conclusion

The burden of diabetic foot ulceration is immense and will continue to grow, with estimates of 360 million people suffering from diabetes mellitus twenty years from now. Up to one quarter of people with diabetes will develop a foot ulcer throughout their lives. Currently the risk of this ulcer becoming resistant to conventional therapy and progressing to amputation is excessively high. The cost to healthcare systems worldwide is profound and is accelerated by increasing health care system costs, an aging population, and a rise in the incidence of diabetes and obesity. For the individual with diabetes, lower limb pain, social isolation, physical morbidity, restrictions in work capacity, and psychological well-being all contribute to the burden of a diabetic foot ulcer. The scope of the problem is increased by the ongoing lack of effective treatments of chronic diabetic wounds

The management of the diabetic foot is complex requiring a multidisciplinary approach. Chapter 1 reviews the current standards of care required for investigation, treatment and prevention of diabetic foot ulceration and consequent amputation. The focus of this research is on neuropathic foot ulceration. Current conventional treatment strategies result in 31% of diabetic neuropathic ulcers healing at 20 weeks and 24% healing at 12 weeks.[1] These data highlight the suboptimal treatment of diabetic ulceration and the critical clinical need to develop novel therapies. The aim of this research is to develop novel 'biological dressings' to treat non-healing diabetic foot ulcers using tissue engineering and regenerative medicine strategies. The goal of treatment is to reduce time to wound closure.

Stem and Progenitor Cell Therapy for Wounds
The biology of diabetic cutaneous wounds is reviewed in chapter 2. The pathological processes in non-healing diabetic wounds arise from chronic inflammation and impaired angiogenesis. Conventional wisdom has been that growth factors are central to effective wound healing. Specific growth factors have been investigated over 3 decades with limited clinical benefit. The ultimate solution to under-healing is likely to be administration of cells that retain the ability to elaborate the full complexity of biological signalling, together with the environmental cues that are needed to regulate

150

the differentiation and proliferation of these cells.[2,3] Currently available somatic cell therapies have demonstrated only modest clinical benefit. Stem and progenitor cells, specifically circulating angiogenic cells or endothelial progenitor cells and mesenchymal stem cells are potentially superior cell types and are positioned as perfect treatment modalities for correcting the defective wound healing associated with diabetes. As reviewed in chapter 2, basic science demonstrates evidence that circulating angiogenic cells and mesenchymal stem cells represent cell types that may treat the pathology associated with delayed diabetic wound healing. In this research, the wound healing effect of topical administration of autologous circulating angiogenic cells (CACs) and allogeneic mesenchymal stem cells (MSCs) was investigated.

Animal Model of Diabetic Wound Healing

The proof of efficacy of novel treatments, require testing in a preclinical model. The complexity of human diabetic foot ulceration with its multi-factorial pathology cannot be fully realised in an animal model. The most robust clinically relevant wound healing endpoint is percentage wound closure. The rabbit ear dermal ulcer model is a powerful model for examining ulcer healing in an excisional wound.[4] This model was chosen as healing occurs in a more similar pattern to human cutaneous ulcers. Ulcer healing does not heal by contraction as occurs in other rodent models. In chapter 3, the alloxan-induced diabetic rabbit ear ulcer model is validated. After 5 weeks of hyperglycaemia, a significantly increased percentage wound closure is present in non-diabetic animals as compared to diabetic animals, indicating that this model is a compromised wound, allowing investigation of novel treatments.

Circulating Angiogenic Cells

The discovery and ongoing investigation of this cell type has illuminated the field of vascular biology and more specifically diabetes-related vascular dysfunction. The CAC is readily isolated from peripheral blood and is an attractive autologous topical treatment for diabetic ulcers in humans. Chapter 2 reviews the current evidence and scientific background to CACs or early endothelial progenitor cells. Diabetes Mellitus results in CAC dysfunction which maybe rescued by ex-vivo manipulation of cells.

Our group have reported that CAC dysfunction is reversed by exposure to osteopontin, a matri-cellular protein. The benefit of ex-vivo manipulation of circulating angiogenic cells using osteopontin is proven in a model of hindlimb ischaemia.[5] This research assesses the benefit of autologous CACs exposed to osteopontin in cutaneous wound healing.

Diabetes Mellitus leads to dysfunction in CAC mobilisation and homing to cutaneous wounds. Systemic delivery of cells results in cells dying after transplantation. Topical delivery of CACs to a wound overcomes these hurdles and this was the cell transplantation methodology used in experiments. In chapter 3, a cell-scaffold treatment was successfully developed by seeding CACs in a type 1 bovine collagen scaffold.

Subsequently, the hypothesis that autologous diabetic CACs which are exposed to osteopontin ex vivo and transplanted to a full thickness cutaneous wound augment wound healing was tested. The percentage wound closure was significantly greater in wounds treated with CACs exposed to osteopontin and seeded on a collagen scaffold, as compared to wounds treated with CACs seeded on collagen, wounds treated with collagen alone or untreated wounds. Extensive stereological analysis of wounds indicates a more efficient neovasculature in the wounds treated with CACs exposed to osteopontin and delivered using a collagen scaffold. There is an increased blood vessel diameter with a reduced radial diffusion distance in wounds treated with CACs exposed to osteopontin and delivered via a collagen scaffold as compared to untreated wounds.

The mechanism of action of CACs is through paracrine effect. To further investigate the diabetes-related CAC dysfunction, the secretome of human diabetic and non-diabetic CACs was examined in chapter 4. Non-diabetic CAC conditioned media is superior to diabetic CAC at supporting angiogenesis as evident from the matrigel assay. The exact mechanism was not evident from this study as there was no significant difference found in the levels of angiogenesis-related peptides between diabetic and non-diabetic groups.

Mesenchymal Stem Cells

Research on mesenchymal stem cell (MSC) treatment is at a more advanced stage than CAC research. There exists a growing body of evidence on MSC therapy in the treatment of human disease. Safety and efficacy has been assessed with diseases including ischaemic heart disease and graft versus host disease with favourable outcomes. MSCs are pluripotent adult derived stem cells. Chapter 2 reviews the benefit of mesenchymal stem cells in the treatment of diabetic ulcers. In the published literature, autologous topically applied MSCs have been used to treat diabetic ulceration in 10 humans with beneficial effect. MSCs are immunoprivileged, and exert their beneficial effect by differentiation into other cell types and by paracrine effect. As evident from autologous CAC experiments, there is dose limitation with the diabetic condition. However MSCs proliferate readily in culture and there is no dose-limiting effect associated with these cells. Allogeneic transplantation is possible and cells maybe cryopreserved and used as a potential 'off-the-shelf' product.

MSCs increase cutaneous wound healing by a number of mechanisms as described in chapter 2. These include differentiation into keratinocytes. MSC increase the blood supply in wounds via paracrine means. MSCs suppress inflammation and are suitable for allogeneic transplantation. Recent evidence describes the effect of MSCs in the benefiting host defence. MSCs appear to function as a critical fulcrum, providing balance by promoting pathogen clearance during the initial inflammatory response while suppressing inflammation to preserve host integrity and facilitate tissue repair.[6]

The wound healing benefit of non-diabetic allogeneic bone-marrow derived MSCs was assessed. MSCs were delivered via a collagen scaffold using a dose-escalation treatment protocol. The same diabetic wound healing model as was used for CACs was used to assess treatment efficacy. Accelerated wound healing was observed using treatment with 1,000,000 MSCs seeded on a collagen scaffold as compared to untreated wound. This treatment demonstrated increased percentage wound closure as compared to control. This wound healing benefit was not seen with doses of 50,000 or 100,000 cells. Extensive stereological analysis revealed statistically longer blood vessels with a reduced radial diffusion distance in wounds treated with 1,000,000 MSCs seeded in a collagen scaffold. This demonstrates a more efficient

neovasculature in these wounds. Inflammation was not increased in MSC and collagen treated wounds as compared to untreated wounds supporting the concept of an immunomodulatory benefit from MSC treatment. This study provides evidence of the wound healing benefit of allogeneic non-diabetic bone-marrow derived MSCs in the treatment of diabetic ulcers.

In summary, stem and progenitor cell therapy augment wound healing. Topically-applied circulating angiogenic cells exposed to osteopontin and seeded on a collagen scaffold significantly increase percentage wound closure as compared to wounds treated with CACs not exposed to osteopontin on collagen, wounds treated with collagen alone and untreated wounds. Allogeneic non-diabetic bone marrow derived MSCs delivered using a collagen scaffold augment wound healing in the same preclinical model. The wound healing benefit of MSCs is dose dependent. The wound healing benefit of both cell types is associated with the development of a more efficient neovasculature in a highly relevant preclinical animal model.

Several questions of interest remain, Which cell type is more effective at wound healing?, Is co-transplantation of both cells superior than either alone?, What is the effect of repeated treatments?. This warrants further research and the preclinical model allows for these questions to be addressed. Autologous therapy is potentially safer than allogeneic therapy, and subject to less regulatory constraints, yet MSCs have been used in treatment of human diseases in the past with a good safety profile. In the first instance, in light of the critical clinical need to develop novel therapies to increase time to complete wound healing and prevent amputation, the results of this research require translation to human trials.

Bench-to-Bedside: Translating Cell Therapy to Humans.

The importance of translational science is being recognised as a key driver to the realisation of basic science discoveries for humans. The scientific basis for potential success with topical stem and progenitor therapy in treatment of human diabetic foot ulcers is presented. This research has proven the efficacy of topical treatment with both CACs exposed to osteopontin and mesenchymal stem cells. The success of these treatments in humans will rely on clinical efficacy, safety, ease of use and cost-effectiveness. This information can only be obtained as part of a clinical trial. Stem

and progenitor cell-based topical treatments will not be used in isolation to treat diabetic foot ulceration. These advanced biological treatments will be part of a treatment algorithm, which would see the implementation of standard care initially to wounds and if this fails, the institution of cell treatment as rescue therapy.

With any new cell-based therapy, it is mandatory to ensure safety for the patient. Any negative toxic side-effect of cell-based therapies would be a set back for the field of tissue engineering and regenerative medicine. In Europe, the European Medicines Agency (EMA) controls regulation and clinical trials of new cell based products. This preclinical research provides the evidence required for the application and undertaking of a human clinical trial.

The development of topically delivered cell therapy to treat human diabetic foot ulcers require that the cells be produced in accordance with good manufacturing practice guidelines. Before application to patients, stringent release criteria for a cellular therapy product must be met. This ensures identity of the cells by immunophenotypical characterisation, cell viability testing, ensuring no growth for infectious organisms and no karyotype abnormalities of cells. Once the stem and progenitor cells have fulfilled release criteria, they will be seeded on a collagen scaffold. The collagen scaffold will be of clinical grade collagen. A potentially suitable biomaterial is Integra™, a currently available type 1 collagen product, licensed for use in humans. Injection of cells onto the collagen scaffold will be by direct injection as performed in preclinical experiments. The size of cell scaffold will be determined by the size of the ulcer to which it is to be applied. The seeding density will be the same as used in the preclinical experiments described in chapters 3 and 5.

A phase 1 safety study will be performed to assess safety and efficacy of the topically applied MSC therapy. This will be preformed after appropriate ethical and regulatory approval has been obtained. A dose escalation protocol will be assessed using 3 increasing doses as performed in preclinical experiments. Each dose will be applied to 3 patients, with a total of 9 patients receiving cell treatments. The treatment will be applied to ulcers, which are not healing after 4 weeks of conventional therapy.

Finally, the potential to translate this novel cell therapy to a variety of dermatological disorders increases the attractiveness of stem and progenitor cell therapy for the management of other chronic diseases. These include but are not limited to decubitus ulcers and burns. Stem and progenitor cell therapy is a new field encompassing both tissue engineering and regenerative medicine science and holds promise for the improved treatment of diseases which are sub-optimally managed with current therapies

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