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

Aonghus O’Loughlin¹, Mangesh Kulkarni², Michael Creane¹, Erin Vaughan¹, Emma Mooney¹, Georgina Shaw¹, Mary Murphy¹, Peter Dockery³, Abhay Pandit², Timothy O’Brien¹

1. Regenerative Medicine Institute, National Centre for Biomedical Engineering and Science, National University of Ireland, Galway, Galway, Ireland
2. Network of Excellence for Functional Biomaterials, National University of Ireland Galway, Galway, Ireland
3. Department of Anatomy, National University of Ireland, Galway, Ireland

Corresponding Author: Timothy O’Brien

Address: Regenerative Medicine Institute,
National Centre for Biomedical Engineering and Science,
National University of Ireland, Galway,
Galway City
Ireland

Telephone: 00353 91 495166
Fax: 00353 91 495547
E-mail timothy.obrien@nuigalway.ie

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Abstract

There is a critical clinical need to develop therapies for non-healing diabetic foot ulcers. Topically applied mesenchymal stromal cells (MSCs) provide a novel treatment to augment diabetic wound healing. A central pathological factor in non-healing diabetic ulcers is an impaired blood supply. It was hypothesized that topically applied allogeneic MSCs would improve wound healing by augmenting angiogenesis. Allogeneic non-diabetic bone-marrow derived MSCs were seeded in a collagen scaffold. The cells were applied to a full thickness cutaneous wound in the alloxan-induced diabetic rabbit ear ulcer model in a dose escalation fashion. Percentage wound closure and angiogenesis at 1 week was assessed using wound tracings and stereology respectively. The topical application of 1,000,000 MSCs on a collagen scaffold demonstrated increased percentage wound closure when compared to lower doses. The collagen and collagen seeded with MSCs treatments result in increased angiogenesis when compared to untreated wounds. An improvement in wound healing as assessed by percentage wound closure was observed only at the highest cell dose. This cell-based therapy provides a novel therapeutic strategy for increasing wound closure and augmenting angiogenesis which is a central patho-physiological deficit in the non-healing diabetic foot 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. 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. MSCs provide a novel therapeutic treatment and have been shown to be beneficial in diabetic wound healing (3). The mechanisms underlying the beneficial effect of wound healing 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. MSCs can be delivered in an allogeneic fashion, and possess immunosuppressant and immunomodulatory properties (4).

To date, there have been encouraging results in preclinical models of diabetic wound healing. Treatment with MSCs resulted in increased wound closure, new granulation tissue formation and increased blood vessel formation and cellularity (5-8). In addition, 10 humans have received autologous mesenchymal stem cells resulting in augmented wound healing. There is one report of an effect related to dose with autologous MSCs seeded in a fibrin spray (9). Nonetheless, there have been no studies using allogeneic human MSC transplantation in the setting of diabetic cutaneous ulceration. There is a paucity of data on effective dosing strategies in the literature. In this study, we report for the first time a dose response evaluation of allogeneic transplantation of MSCs delivered through a collagen scaffold to an ulcer in a diabetic animal model.

Previous research has shown that infusions of several cell types into the body rapidly undergo cell death (10). After intravenous delivery, MSCs are found at low or very
low frequencies in target organs (11). The use of biomaterials in conjunction with stem cell therapy in vivo may ensure sustained viability and functionality of cells. (10). Collagen supports angiogenesis (12). A biomaterial such as collagen allows targeted delivery and positioning of high numbers of cells at the wound site. It was hypothesised that topical application of a collagen scaffold seeded with allogeneic non-diabetic bone marrow derived MSCs would support angiogenesis and augment cutaneous wound closure in a diabetic animal model of cutaneous ulceration. The therapeutic effect of collagen seeded MSC therapy in a pre-clinical model using wound tracings and stereology was investigated. This technique is a scientifically robust validated strategy to assess in vivo tissue responses to tissue engineered constructs.

**Research Design and Methods**

**MSC Culture and Characterisation**

*In vivo* experiments were carried out under a licence from the Department of Health, Ireland and the National University of Ireland Galway Institutional Animal Use Committee. Allogeneic bone marrow derived mesenchymal stem cells were isolated from healthy rabbit bone marrow as previously described (12). Briefly, the animal was euthanized and bone marrow MSCs were isolated using collagenase digestion. Bone marrow aspirates were washed with Dulbecco’s phosphate buffered saline (Sigma-Aldrich, Ireland) and precipitated mononuclear cells were suspended in MSC culture medium. Cells were grown in alpha MEM media (Gibco, Invitrogen, CA, USA) supplemented with 10% foetal bovine serum (PAA Laboratories Ltd., UK) and 5% penicillin/streptomycin. Cells were maintained at 37°C and 95% humidity and 5% CO₂ in the same medium. Non-adherent cells were washed off after 5 days and fresh medium added. Colonies formed after 9 days and were trypsinized after 60-90% coverage with 0.25% trypsin/0.53 mM ethylenediamine tetraacetic acid (Sigma). Under appropriate culture conditions, differentiation assays were performed to confirm cell differentiation into chondrogenic, osteogenic and adipogenic lineages (13). 200,000 MSCs aliquots were frozen in liquid nitrogen at passage 3 and these cells were used for future experiments.
**Fabrication of Collagen Scaffold and Cell Seeding**

Type 1 bovine collagen solution was isolated and purified as described previously (14). 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 lyophilized using a VirTis freeze-dryer (Suffolk, U.K). After washing in HANKs ( sigma), 70% ethanol, sterile water and media, 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, Thermo Fisher Scientific, Denmark) After 4 days, confluent MSCs were trypsinized and seeded by injecting cells in 1000 µl of alpha MEM supplemented media using an insulin syringe (BD, UK) Cells were placed in an incubator for 16 hours in 37°C and 5% CO₂. 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 alamarBlue™ (rezasurin) (Invitrogen). 24 hours after seeding, the cells were washed once in Hanks balanced salt solution (Sigma) and incubated for 3 hours in 10% alamarBlue™. 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 alamarBlue™ was determined as previously described (15). In one animal experiment MSCs were labeled using PHK-26 (Sigma-Aldrich, Ireland) and DAPI nucleic acid stain (Sigma-Aldrich, Ireland) according to manufacture’s instructions and the cells were imaged using a fluorescent microscope (Olympus).

**Scanning Electron Microscope**

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

Nine male NZW rabbits (3-3.5Kg) were used in the study. The animal were outbred, 3-6 months old and purchased from Harlan Ltd., UK. 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, with a 12 hour light/dark cycle and controlled temperature and humidity. Rabbits were fed a standard chow and water *ad libitum*.

**Induction of Hyperglycemia**

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, Ireland) 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 hypoglycemia. Serum blood glucose was checked daily using Accucheck™ advantage strips (Roche, USA). Insulin therapy was administered if the animal lost weight and had ‘high’ (greater than 33 mmol/L) glucose readings using insulin glargine (Sanofi-Aventis, Ireland).

**Surgical Procedure**

After 5 weeks of hyperglycemia, rabbits were anaesthetized using intramuscular injection of 0.1 mL/Kg xylazine and 0.12 mL/Kg of ketamine. 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™, Smith & Nephew, UK) and the ear was stitched and covered with adhesive dressing (Operfix™, Promedicare, Ireland), 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 (16). 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 one week was calculated using formula A (Formulae are presented in supplementary data).

**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, Houston, USA) 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 haematoxylin and eosin, and Masson’s trichrome using standard protocols.

**Wound Volume**

Wound Volume was calculated by multiplying the average wound thickness by the area of the wound tracing one week after wounding (Figure 1).

**Stereology**

Stereology is a means of assessing tissue responses to tissue constructs (17). It allows assessment of angiogenesis in vascular beds (18). In this present study, vertical sections of the tissue were examined using a systematic random sampling strategy to provide estimates of relevant stereological parameters (19). Methods used to measure the length of vessels in three dimensions were based using a vertical orientation design and a cycloid test system (20). A series of cycloid lines were placed on the histology sections using Image Pro Plus™ software (Media Cybernetics, Maryland, USA) as previously described (16). 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 (16). The fields were captured at 40X 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 ($L_v$), which is based on the principle that the longer and more convoluted a vessel, the greater the number of occasions its profile intersects a plane (16, 17).

Length density and surface density of blood vessels were analyzed with and without multiplying by the wound volume. The surface density of blood vessels was calculated using formula B 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 C. 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 D. 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 (21).

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 (16). Inflammatory cells were counted and included lymphocytes and neutrophils. This was counted using a 192 point grid using Image Pro Plus™ software (Media Cybernetics, Maryland, USA). Neutrophils were identified as small dense circular multi-lobed cells and lymphocytes as small round dense cells with large nuclei. Formula F calculates inflammatory cell infiltrate in tissue.
Statistics
Analysis between groups was assessed using Analysis of Variance and post hoc analysis with Fisher’s Pairwise comparison. P < 0.05 was taken as significant. Minitab software was used. Bar graphs represent mean ± standard deviation.
Results

Induction of Diabetes Mellitus in the Animal Model

The animals remained hyperglycaemic post alloxan infusion over the study time period (Supplementary Figure 1). There was no mortality post alloxan treatment. Two animals required insulin administration after alloxan treatment. Insulin therapy was administered if the animal lost weight and had high glucose readings using insulin glargine (Sanofi-Aventis, Ireland). High glucose readings were indicated on the glucometer as ‘high’ and signified serum glucose of greater than 33 mmol/L. The animal was administered 6 units of insulin subcutaneously if this occurred and the blood glucose was monitored 12 hours later.

MSC Culture and Characterization

MSC were successfully isolated from non-diabetic New Zealand White rabbit bone marrow. Cells were cultured to passage 3 and frozen in liquid nitrogen in 200,000 cell doses. When ready for use, cells were thawed and plated on tissue culture plastic. MSCs on tissue culture plastic demonstrated spindle shaped morphology on becoming confluent. MSCs differentiated into chondrocytes, osteocytes and adipocytes when exposed to appropriate conditions (Supplementary Figure 2). Cell surface immunophenotyping was not carried out due to the lack of rabbit specific antibodies available.

Metabolic Activity of Cells

MSCs were seeded on collagen. After seeding the cells on collagen, metabolic activity was assessed at separate time-points up until two weeks in vitro (Supplementary Figure 3).

Histology and Scanning Electron Microscopy

After immersion fixation, tissue processing and sectioning of the MSC-collagen constructs, haematoxylin and eosin and Masson’s trichome staining was performed. Cells were predominantly located on the superior border of the collagen scaffold (Supplementary Figure 4). Scanning electron microscopy images (Figure 2, A-D) revealed densely populated MSCs within the collagen scaffolds seeded with 1,000,000 cells.
Detection of Transplanted MSCs in the Wound
Supplementary figure 5 demonstrates PKH-26 labelled rabbit MSCs in the wound one week post treatment. MSCs were present in the wound for at least seven days and the collagen scaffold was successful in mediating cell delivery to the wound.

Histology
Figure 3 illustrates representative samples of Masson’s trichrome 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 demonstrate increased epithelial and granulation tissue formation in collagen seeded with MSC treatment group as compared to untreated wound and wounds treated with collagen alone. This benefit is observed in collagen seeded with MSCs groups at all of the treatment doses administered.

Percentage Wound Closure
Transplantation of 1,000,000 rabbit MSCs seeded on a collagen scaffold resulted in a statistically significant increased percentage wound closure at one week as compared to untreated wounds (Figure 4).

Stereology
Stereological analysis (Table 1) demonstrates significantly increased total length of blood vessels with enhanced neovasculature in wounds treated with 1,000,000 MSCs seeded on a collagen scaffold as compared to untreated wounds. The total length of blood vessels in wounds treated with collagen seeded with 50,000 MSCs, 100,000 MSCs or collagen alone is not significantly greater than untreated wounds.

Blood vessels in collagen treated wounds and collagen seeded with MSCs demonstrate significantly reduced radial diffusion distance when compared to untreated wounds. 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 statistically significant difference in blood vessel diameter between the groups one week after treatment. Figure 5 demonstrates representative images of blood vessels in the untreated wounds and wounds treated with MSC-seeded collagen scaffolds.
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 infiltration in tissue can assess inflammation in wounds one week after treatment (16). No significant difference was observed in inflammatory cell infiltrate between the groups. These data provide evidence that the MSC collagen treatment and collagen alone treatment does not result in increased inflammation, as compared to untreated wounds. The clinical relevance of this result is that the treatment does not result in an inflammatory reaction which would potentially result in a reduced healing rate.

The relationship between the various healing parameters was assessed in the treatment groups using Pearson’s correlations (Supplementary Table 2). The significance of the correlation between blood vessel morphology and inflammatory cells may be that the inflammatory cell infiltrate in wounds treated with 1,000,000 and 100,000 MSCs seeded in a collagen scaffold is associated with a more efficient neovasculature and arises from the paracrine effect of higher doses of transplanted MSCs.
Discussion

Topical MSC therapy is a novel treatment for non-healing human diabetic foot ulcers, that are refractory to current standard care. This MSC-seeded biomaterial treatment may reduce amputation rates and alleviate the burden of non-healing diabetic ulcers. A central pathological feature of diabetic foot ulceration is impaired angiogenesis. MSCs are known to promote angiogenesis in addition to improving cutaneous wound healing (7, 22). This research is unique in several aspects: 1, investigating the effect of MSCs in a clinically relevant preclinical model 2, the use of non-diabetic allogeneic MSCs 3, the dose escalation strategy employed and 4, the use of a type 1 collagen biomaterial to mediate cell delivery to the cutaneous ulcer. The results of the research demonstrate that the topical delivery of MSCs to a diabetic wound using a biomaterial augments diabetic wound healing. The wound healing benefit is observed by increased percentage wound closure with an associated more efficient neovasculature.

The animal model of diabetic cutaneous wound healing utilised in this study is a preclinical model, with healing occurring similar to human wound healing. Animal models used in the investigation of topical MSC therapy are predominantly murine, where skin heals by contraction. This is due to the presence of the panniculus carnosus layer which is present in rats and mice, but absent in the rabbit ear and in humans. This functional anatomical layer contracts on wounding. Excisional wounds in the rabbit ear heal by re-epithelialization and new granulation tissue formation as occurs in the human situation (16). Our group has previously described impaired wound healing in the diabetic model used in the current study (16). The duration of hyperglycaemia in the current study was 5 weeks. The wound is a full thickness cutaneous ulcer and facilitates assessment of wound closure and new granulation tissue formation, angiogenesis and inflammation. Using stereological methodology, a comprehensive assessment of host tissue responses to cell seeded biomaterial constructs can be achieved.

This study investigated the therapeutic efficacy of allogeneic non-diabetic bone marrow-derived MSCs delivered to a diabetic wound in an immuno-competent animal. The use of allogeneic MSC transplantation from a non diabetic donor is an approach which may have advantages over autologous cell transplantation in which disease-induced cell dysfunction may limit therapeutic efficacy (23, 24). To facilitate
this approach, cryopreserved cells were used. Non-diabetic bone marrow derived MSCs remained viable after freezing in liquid nitrogen and differentiated into three mesodermal cells, adipocytes, chondrocytes and osteocytes. MSCs were successfully seeded in the scaffold. The MSC-scaffold treatment retains cellular viability in-vitro. This provides evidence for the use of non-diabetic MSCs which are frozen in liquid nitrogen as an “off-the-shelf” product.

The animals used in the study were outbred, however it is possible that genetic variability within the rabbit colony for Major Histocompatibility Complex (MHC) proteins could be limited and, as such, might resemble more closely a cell transplant between related individuals than between unrelated, fully Major Histocompatibility mismatched individuals. The implication being that immunogenicity based on MHC disparity could be lower than that which would apply following fully mismatched cell delivery. However, we demonstrate in this paper that cells isolated from non-diabetic animals can be successfully transplanted to diabetic animals following cryopreservation.

As the body of data from pre-clinical research investigating the cutaneous wound healing benefit of MSCs is increasing, the positive results in preclinical studies will require the focus to switch to the translation of MSC based therapy in human subjects (25). A majority of studies have investigated direct injection of cell suspensions around the wound but little is known of cell engraftment or retention at the wound site. It is known that cells injected directly into the body undergo cell death rapidly (10). Biomaterials may support cell viability and thus enhance therapeutic efficacy. Topical delivery of MSCs reduces the potential toxicity associated with systemic administration. One previous report demonstrates 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 (26). MSCs were injected intradermally around the cutaneous wounds in diabetic rats. Angiogenesis endpoints in histological sections were assessed using similar stereological methodology as used in our study. In contrast to the results of our current study, this study demonstrated MSC augmented wound healing which was not associated with increased angiogenesis (26). Collagen is a natural biomaterial which promotes sustained cellular viability and functionality in addition to maintaining the cells at the wound site (9,10) and was used
to deliver MSCs to the wound surface in our study. Collagen is known to support angiogenesis when used alone (12).

The study investigates an allogeneic strategy, using a dose escalation regimen. In this dose escalation study of topical MSC therapy, transplantation of 1,000,000 cells on a collagen scaffold revealed increased percentage wound closure when compared to untreated wounds. 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 (27). This study reviewed extensive histological sections 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 cell group. Both surface density and length density 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. In addition, the total length of blood vessels in the wound was significantly greater in wounds treated with collagen seeded with 1,000,000 MSCs as compared to other groups. 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. It should be acknowledged however that the translation of this information to human trials is not completely clear e.g. should the dose chosen for phase 1 human studies be based on wound area or body weight in rabbits versus humans.

The stereological analysis and comparison between groups revealed no difference in inflammatory cell infiltrate between treatment groups and untreated wounds. This is an important observation to ensure that the tissue engineered construct does not illicit an inflammatory 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 collagen and collagen seeded with MSCs transplantation. Collagen seeded with 1,000,000 MSCs resulted in a significantly increased percentage wound closure and a superior
vascular supply when compared to untreated wounds at one week. This is the first extensive analysis of MSCs delivered to a wound using a collagen scaffold in the context of diabetes mellitus. It confirms that the wound healing benefit of MSC transplantation on a collagen scaffold 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 (22).

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Professor Timothy O’Brien is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. TOB, AOL, AP designed the study. AOL, MK, MC, GS, EM, MM performed the experiments. AOL, MK, EEV, AOL, PD, AP and TOB analyzed the data AOL, TOB, AP, MK wrote the manuscript. The abstract was previously published at the American Diabetes Association meeting Philadelphia 2012.

Conflicts of Interest

Timothy O’Brien is a founder, director and equity holder in Orbsen Therapeutics Ltd. For all other authors, there exists no conflict of interest.
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Legends for Figures and Tables

Figure 1. Example of cross sectional image of Wound stained with Haematoxylin and Eosin. Scale Bar = 1mm. Six measurements were taken from the cartilage to the wound surface and measured using Cell B™ software (Olympus), and the average thickness calculated. The average thickness was used to calculate wound volume which is utilized for calculation of stereological endpoints.

Figure 2. 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 dose of 1,000,000.

**Figure 3.** Masson’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. Scale bar = 1 mm. There appears to be a dose dependent increase in the epithelialization over the three doses. In the representative images of wounds depicted in Figure 3, there is the subjective appearance of increased new granulation tissue in the wound and a more organized wound healing response in wounds treated with collagen-seeded MSCs in comparison to untreated wounds and wounds treated with collagen alone. (Figure 3A-3F).

**Figure 4.** Percentage wound closure of cutaneous ulcers one week after treatment with MSCs seeded in a collagen scaffold. Analysis between groups using ANOVA and Fisher’s Pairwise comparison. *P<0.05. Error bars = Standard Deviation. 1,000,000 MSCs seeded on a collagen scaffold results in significantly increased percentage wound closure as compared to control. There was no observed difference in observed percentage wound closure between the other treatment groups i.e. collagen + 50,000 MSCs, collagen + 100,000 MSCs or collagen alone when compared to untreated wounds. This result supports the hypothesis that the wound healing effect of MSC and collagen treatment occurs in a dose dependent fashion. Increased cell doses increase percentage wound closure and rate of wound healing.

**Figure 5.** Representative images of neovasculature in control wounds (A), and wounds treated with 1,000,000 MSCs seeded in a collagen scaffold (B). Tissue samples are fixed in paraffin at 5 micrometer depth and stained with haematoxylin and eosin. Scale bar = 200 micrometers. An increased blood vessel density and reduced radial diffusion distance is evident in the wounds treated with MSCs and
collagen as compared to untreated wounds is evident in Figures 5B and 5A respectively.

**Table 1.** Stereological analysis of wounds in diabetic animals. (Analyzed by ANOVA followed by Fisher’s Pairwise comparison, n=9, *P<0.05, #P<0.05 compared to untreated wound. (± Standard Deviation) The surface density of blood vessels in wounds treated with collagen and collagen seeded with MSCs at all doses was significantly increased as compared to controls. This indicates a significantly increased area of blood vessels present in the wound to ensure an increased area of capillaries available for gaseous exchange. In addition, 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 wounds demonstrated longer more convoluted vessels as compared to untreated wounds. This vasculature is more efficient than that observed in 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.
# Treatment Groups

<table>
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<th>Parameter</th>
<th>1 x10^6 MSCs + Collagen</th>
<th>100,000 MSCs + Collagen</th>
<th>50,000 MSCs + Collagen</th>
<th>Collagen</th>
<th>Untreated Wound</th>
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<tr>
<td>Volume of Inflammatory Cells (mm³)</td>
<td>3.6 ± 1.9</td>
<td>3.6 ± 1.6</td>
<td>3.5 ± 0.9</td>
<td>3.1 ± 1.1</td>
<td>2.4 ± 1.0</td>
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<td>Surface Density of Blood Vessels in Wound (1/mm)</td>
<td>56.8 ± 20.1*</td>
<td>53.3 ± 9.8*</td>
<td>52.7 ± 10.7*</td>
<td>46.2 ± 11.2*</td>
<td>31.0 ± 6.8</td>
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<td>Surface Area of Blood Vessels (mm²)</td>
<td>1393 ± 781</td>
<td>1094 ± 409</td>
<td>1196 ± 345</td>
<td>1135 ± 378</td>
<td>929.6 ± 459</td>
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<tr>
<td>Length Density of Blood Vessels in Wound (mm²)</td>
<td>11140 ± 3737*</td>
<td>11264 ± 2394*</td>
<td>10969 ± 2312*</td>
<td>9627 ± 2711*</td>
<td>5425 ± 1591</td>
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<tr>
<td>Total Length of Blood Vessels in Wound (mm)</td>
<td>270731 ± 146549*</td>
<td>231894 ± 90588</td>
<td>250521 ± 80213</td>
<td>234213 ± 75625</td>
<td>162924 ± 90070</td>
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<td>Radial Diffusion Distance (microns)</td>
<td>5.6 ± 0.1*</td>
<td>5.4 ± 0.7*</td>
<td>5.5 ± 0.8*</td>
<td>5.9 ± 0.8*</td>
<td>7.9 ± 1.3</td>
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<td>Vessel Diameter (microns)</td>
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<td>1.5 ± 0.2</td>
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Supplementary Figure 1. 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.
Supplementary Figure 2A. Differentiation assays of rabbit MSCs. These images demonstrate differentiation into adipocytes (oil red O) and osteocytes (Alizerin Red, Alkaline Phosphatase and Von Kossa). Control cells are in the column on the left and treated cells in the right column. (Supplementary figures 2a and 2b) depict results of differentiation assays and confirm the ability of non-diabetic rabbit bone marrow derived MSCs to differentiate into osteogenic, chondrogenic and adipogenic lineages.
**Supplementary Figure 2B.** Rabbit MSCs demonstrate differentiation into chondrogenic lineage as evidenced by increased amount of glycosaminoglycan (GAG)/DNA in MSCs at 14 and 21 days.

**Supplementary Figure 3.** Time-course of Metabolic Activity of MSCs seeded on a collagen scaffold. Metabolic activity is assessed using Alamar Blue (resazurin) (Invitrogen). Rabbit MSCs retained metabolic activity after seeding for 24 hours. 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.
Supplementary Figure 4. Five micrometer histological sections of collagen scaffold seeded with 1,000,000 rabbit MSCs. **A.** Masson’s trichome stained section (2X magnification) Scale bar = 1 mm. **B.** Masson’s trichome stained section 10X magnification. Scale bar = 200 micrometers. MSCs are located predominantly on the superior surface of the collagen scaffold. MSCs are stained purple and collagen stained green.
Supplementary Figure 5. Fluorescently labeled 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 Labeled MSCs and DAPI stained cells. D. Overlay bright-field image and labeled MSCs (red) and DAPI stained cells (blue). This provides evidence that the cells were located in the wound site at one week post treatment. The cells dual-stained with DAPI, a nuclear stain confirming dual-stained cells are transplanted MSCs. This observation demonstrates that MSCs were present in the wound for at least 7 days and that the collagen scaffold was successful in mediating cell delivery to the wound.
Formulae for Wound Closure and Stereology (15, 16, 20)

**Formula A:** Percentage Wound Closure: \( \% C = \) percentage wound closure, \( A_0 = \) Area of wound at day 0, \( A_i = \) Area of wound at 7 days.

\[
\% C = \frac{A_0 - A_i}{A_0} \times \frac{100}{1}
\]

**Formula B.** Surface Density

\[
S_v = 2 \times \frac{1}{L T}
\]

**Formula C.** Length Density

\[
L_v = \frac{(2 \times I L)}{T S}
\]

**Formula D.** Radial Diffusion Distance

\[
R_{\text{diff}} = \frac{1}{\sqrt{\pi} \times L_v}
\]

**Formula E.** Blood Vessel Diameter

\[
d = \frac{S_v}{L_v \cdot \pi}
\]

**Formula F.** Inflammation Inflammatory Cell Fraction

\[
= \frac{P_P}{P_T}
\]
### 1,000,000 MSCs + Collagen

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### Collagen

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### Untreated Wound

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Supplementary Table 1
Supplementary Table 1. Pearson’s correlation values between wound healing parameters. WC = Percentage wound closure, I = inflammatory cell infiltrate, Sd = Surface density of blood vessels, Ld = Length density of blood vessels, RDD = Radial diffusion distance. BVD = Blood vessel diameter. TL BV = Total Length of Blood Vessels. *signifies P < 0.05. For wounds treated with 1,000,000 and 100,000 MSCs seeded on a collagen scaffold, the total length of blood vessels positively correlated with inflammatory cell fraction. In the treatment group of 1,000,000 MSCs seeded on a collagen scaffold, inflammatory cell fraction correlates with length density of blood vessels. These correlations were not observed in other groups.