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Review Article

EFFICACY OF MESENCHYMAL STEM CELL INCORPORATION IN DIFFERENT COMPOSITE ELECTROSPUN COLLAGEN NANOFIBERS FOR DIABETIC WOUND HEALING

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ABSTRACT

Currently available skin substitutes for wound healing often suffer from a range of problems including wound contraction, scar formation, and poor integration with host tissue. Engineering skin substitutes by tissue engineering approach has relied upon the creation of three-dimensional scaffolds as extracellular matrix (ECM) analog to guide cell adhesion, growth, and differentiation to form skin-functional and structural tissue. The three-dimensional scaffolds can not only cover wound and give a physical barrier against external infection as wound dressing, but also can provide support both for dermal fibroblasts and the overlying keratinocytes for skin tissue engineering. A successful tissue scaffold should exhibit appropriate physical and mechanical characteristics and provide an appropriate surface chemistry and nano and microstructures to facilitate cellular attachment, proliferation, and differentiation. A variety of scaffolds have been fabricated based on materials ranging from naturally occurring ones to those manufactured synthetically.One specifically highlighted emerging fabrication technique is electrospinning that allows the design and fabrication of biomimetic scaffolds that offer tremendous potential applications in wound healing of skin.Mesenchymal stem cells give rise to both epidermal and dermal cells and can be used with biocomposites and collagen nanofibers for wound healing process.This study is to review the efficiency of composite collagen nanofiber scaffolds *in vitro* culture studies on cell adhesion ,cell proliferation and cell spreading ,and also *in vivo* studies on the wound site of an diabetic animal model.

Key words: Wound Healing, Electrospun collagen scaffold, Mesenchymal Stem cells..

INTRODUCTION

Stem cells differ from other kinds of cells in the body. All stem cells regardless of their source have three general properties: they are capable of dividing and renewing themselves for long periods; they are unspecialized; and they can give rise to specialized cell types. Unlike muscle cells, blood cells, or nerve cells which do not normally replicate themselves stem cells may replicate many times, or proliferate. A starting population of stem cells that proliferates for many months in the laboratory can yield millions of cells. If the resulting cells continue to be unspecialized, like the parent stem cells, the cells are said to be capable of long-term self-renewal. One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. For example, a stem cell cannot work with its neighbors to pump blood through the body (like a heart muscle cell), and it cannot carry oxygen molecules through the bloodstream (like a red blood cell). However, unspecialized stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells.

Diabetes impairs numerous components of wound healing, including hemostasis and inflammation, matrix deposition, and angiogenesis. These impairments are present in a wide variety of tissues including myocardium, skeletal muscle, nerve, and skin. Cutaneous wounds in diabetes have been shown to have altered blood flow, impaired neutrophil anti-microbial activity, and a dysfunctional inflammatory state associated with abnormal chemokine expression. A number of growth factors essential for wound healing, including FGF-2 and platelet-derived growth factor (PDGF)-B, have also been found to be reduced in experimental diabetic wounds.

In wound regeneration normally epidermis regenerates easily, but when an injury is caused in the skin the dermis and hypodermis do not regenerate at ease(Figure 1). By providing the suitable biomaterials at the wound site, the material might influence the formation of extra cellular matrix in skin. Natural polymers have been used as one of the materials in the scaffolds for soft tissue augmentation. Collagen is one of the predominant protein in extra cellular matrix, used as biomaterial for regeneration of tissue, either skin or bone. The objective of this study is to investigate the use of porous bovine collagen scaffold in the dermal wound healing process [1]

Especially, Protein based biomaterials have the capacity to mimic the extra cellular matrix at the damaged site and also to induce the cell fate process such as cell migration, cell differentiation and cell proliferation. Type I collagen is resorbable, it has high water affinity, low antigenicity, very good cell compatibility and ability to promote tissue regeneration. The use of porous bovine collagen scaffold along with proliferating stem cell in the dermal wound healing process was investigated in this study [2 3 4]

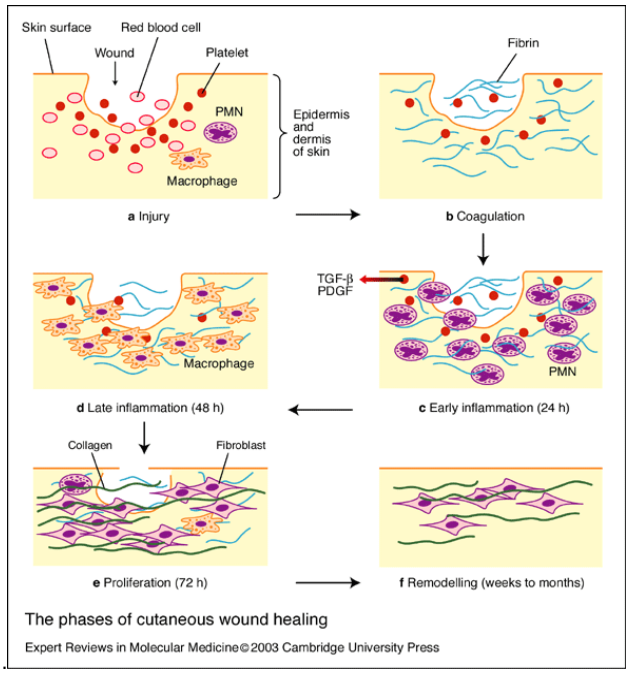
Electrospinning is a versatile technique which recently has significant development in nanofibers production. In this facile technique, a high electric field is applied to the droplet of a fluid which may be a melt or solution coming out from the tip , which acts as one of the electrodes(Figure 2) .This leads to the droplet deformation and finally to the ejection of a charged jet from the tip of the cone accelerating towards the counter electrode leading to the formation of continuous fibers [6]. Due to special characterization of electrospun mat(high surface area to volume ratio, flexibility in surface functionalities, and mechanical properties superior to larger fibers), many research [7-14] have been performed to improve some potential applications of nanofibers including tissue engineering scaffolds, filtration devices, sensors, materials development, electronic applications and etc..

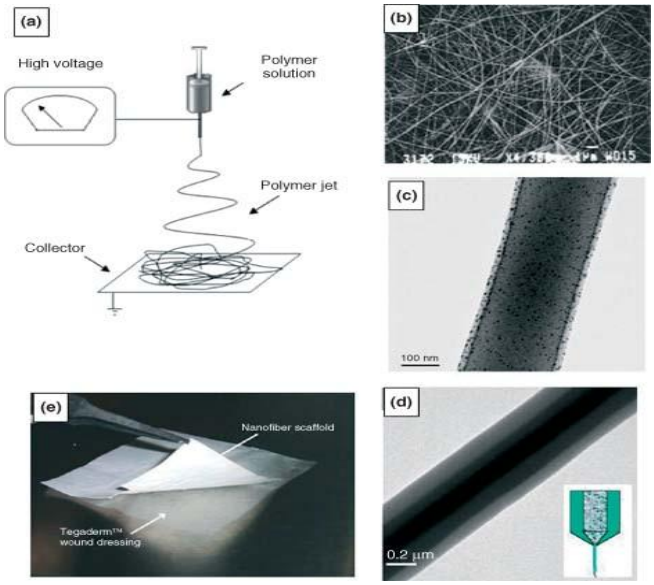
The porous nature of these mats is highly suitable for the drainage of the wound exudates and, allows appropriate permeation to prevent the attack of microbes, thus refraining the wound from possible infections, which would ultimately result in delayed healing atmospheric oxygen to the wound. For successful tissue regeneration, the cells constituting the tissues to be regenerated are necessary. Considering the proliferation activity and differentiation potential of the cells, stem cells are practically promising. The interest in adult stem cells has particularly been triggered by the numerous ethical dilemmas surrounding the use of embryonic stem cells in preclinical and clinical studies [15]. The aim of this review paper is to discuss and summarize the basic principles pertinent to the healing of a wound and to give an overview of recent developments in seeding mesenchymal stem cells on wound dressing materials with an emphasis on electrospun polymeric membranes used for this purpose. Standard tests for characterizing parameters are discussed with relevant methods of performing the test.

Chemicals and reagents: Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum (FBS), glutamine, antibiotics and

Trypsin-EDTA solution, 2mm L-glutamine (Gibco,USA), 100 u/ml penicillin (Sigma) and 100 u/ml streptomycin, Trypsin.

Figure 1: Phases of wound Healing



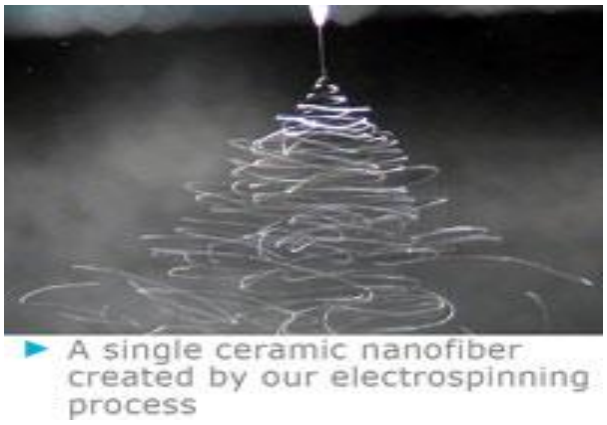


Type I Collagen, Electro spinning Unit.

Isolation and expansion of MSCs

Balb/c Mice, 6-8 weeks old, were sacrificed by cervical dislocation and their femurs and tibiae were carefully cleaned from adherent soft tissue. The tip of each bone was removed with a rongeur, and the marrow was harvested by inserting a syringe needle (27-gauge) into one end of the bone and flushing with Dulbecco's Modified Eagle's Medium (DMEM). The bone marrow cells were filtered through a 70-mm nylon mesh filter . Cells were plated into 6-well plastic cell culture plate at a density of 25 X 10^6 cells per well in DMEM containing 15% fetal bovine serum (FBS; Sigma), 2mm L-glutamine, 100 u/ml penicillin and 100 u/ml streptomycin. Cultures were kept at 37ºC in a humidified atmosphere containing 95% air and 5% CO_2.When primary cultures became nearly confluent, the culture was treated with 0.5ml of 0.025% Trypsin containing 0.02% EDTA for 2 minutes at room temperature. The cells which were lifted within 2 minutes were harvested and cultured in a 25 cm^2 flask. Once the culture reached 70-80% confluence, the cells were harvested for further experiments. Furthermore, the cells not lifted after two minutes of trypsin treatment were kept in culture media for two weeks, at which point these cells were collected for further analysis.In addition, some bone marrow cells were cultivated without frequent medium changes (WFMC cells) as a cell culture control, WFMCs were cultured in 6-well culture plates at a density of 25 X106 cells per well in DMEM and the culture medium was changed after 72 hours for the first time. Cell cultivation was continued for two weeks at which time the cells were analyzed.

Fabrication and preparation of PCL/collagen/PES nanofiber scaffold: Type I collagen was dissolved in hexafluoroisopropanol (HFIP) at a concentration of 8% (w/v). PES was dissolved in an organic solvent mixture of N, N, methylformamide and tetrahydrofuran at a final concentration of 15 wt%. The electrospinning setup utilized in this study consisted of three syringes, a ground electrode (stainless steel drum, 3 and 5 mm outer diameters, 10 cm in length), and a high voltage supply. The distance between the tips and the rotating drum was in the range of 10-25 cm and the positive voltage applied to the polymer solutions was in the range of 15-30 kV(Figure 3 & 4). From the bulk material of the electrospun nanofiber mats, small discs with areas of approximately 0.8 cm^2 were cut out and placed in 24-well cell culture plates. All the scaffolds were soaked in 70 % filtrated ethanol for 2 hour. They were then incubated overnight in DMEM, at 37oC in a humidified 5% CO_2 incubator prior to cell seeding, in order to facilitate cell attachment onto the nanofibers. Now the isolated living MSCs were seeded on to the nanofibers and allowed to grow.



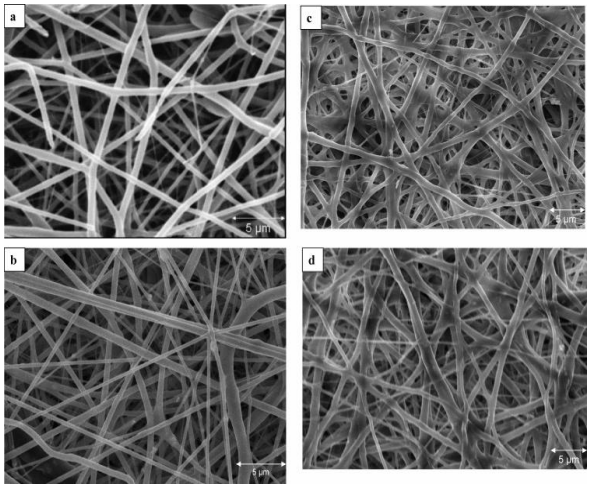


Figure 4: Ranges of electrospun collagen with different porosity.

Colony forming unit-Fibroblast (CFU-F) assay

The clonogenic potential of the isolated cells (first passage) was tested for the colony formation potentials. For this assay, 100 cells were plated on a 60 mm^2 cell culture dish and incubated for 7 days. Subsequently, the plates were stained with 3% crystal violet in methanol for 10 minutes. All visible colonies were counted.

Cell adhesion assay

The crosslinked electrospun composite collagen scaffolds will be placed at the bottom of a 24-well plate. For the assessment of cell adhesion kinetics, human mesenchymal stem cells will be seeded onto the scaffolds(Figure 5).The cells will be allowed to attach to the substrates after seeding in a complete growth culture medium in a CO^2 incubator. The density of attached cells on the scaffolds will be assayed by the MTT assay[16].

Cell proliferation and morphology assay

For cell proliferation study, mesenchymal stem cell will be seeded on a culture plate fixed with the different scaffolds .The proliferation of mesenchymal stem cell on the scaffolds will be quantified after days 1,3, and 7 by MTT assay. The morphology of the adherent cells proliferating on the scaffolds will be examined using SEM. The proliferation of cells was also influenced by the pore size of the nanoporous surface. The nanostructure could control the interaction between extracellular matrix (ECM) molecules and the surface.

Scanning electron microscopy (SEM)

The MSCs grown on the scaffolds were washed with PBS to remove non-adherent cells, fixed in 3% glutaraldehyde for 3 hour at RT and then dehydrated through a series of graded alcohol solutions. After drying, the scaffolds were mounted on aluminum stubs, sputter-coated with gold-palladium (AuPd) and viewed using SEM.

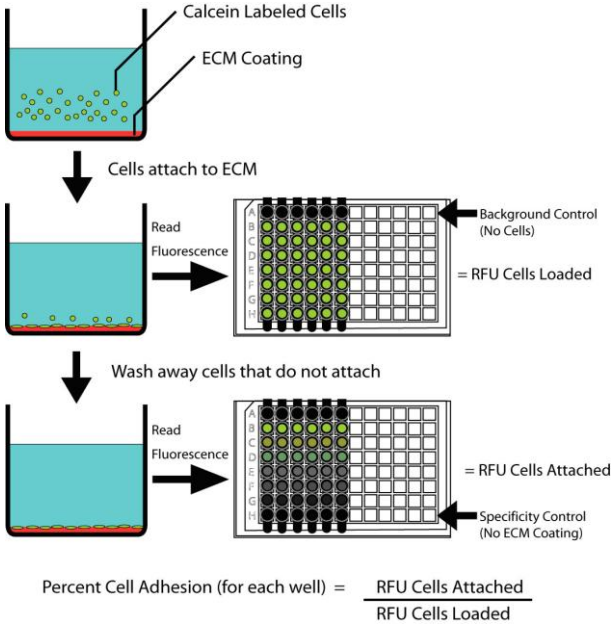


Figure 5: Cell adhesion assay.

Differentiation assays

The potential of the isolated cells to differentiate into osteogenic and adipogenic lineages was examined. For osteogenesis, the cultured cells were incubated in osteogenic conditioned medium[17].DMEM was supplemented with 10 mM β-glycerol phosphate ,50µg/ml ascorbate-2-phosphate and 10^{-7}mM dexamethasone . The culture medium was changed two times per week for up to three weeks. The cells were fixed with methanol for 10 minutes at room temperature and stained with alizarin red with pH4 for 5 minutes at room temperature. Treated cells were subjected to RNA extraction and RT-PCR analysis. For adipogenesis, the cultured cells were incubated in adipogenic medium DMEM supplemented with 50 µg/ml indomethacin,10^{-7} mM dexamethasone (Sigma), and 50 µg/ ml ascorbate-2-phosphate . The culture medium was changed two times per week for up to three weeks. The cells then were fixed in methanol for 45 minutes and stained with Oil Red .

Figure 6: Layering of collagen composites with seeded stem cells on to the wound

In vivo rat wound-healing model

Adult rats [18] with an average weight of 170–200 g can be used.Rats were fasted overnight before surgery, and were anaesthetized by an intraperitoneal injection of a mixture of ketamine (60 mg/kg) and xylazine (9 mg/kg). Dorsal hair was removed with electric clippers, and the skin was disinfected with a povidone-iodine solution. Using a dermal biopsy punch, a surgical wound measuring 8mm in diameter was made by excision of the skin and the panniculus carnosus muscle. Nanoscaffold containing stem cells was placed on to the adjacent skin of the wound. The wound was covered with a transparent semiocclusive dressing [19] to prevent desiccation(Figure 6). The diameter of the wound was measured on days 1, 3, 7, and 10. In addition, histological analyses using hematoxylin & eosin (H&E) staining of wounds were performed 10 days after surgery.

MSCs promote wound healing at in vivo Tissue sections when stained with H&E to resolve the question of whether or not MSCs promote wound healing in skin. Edematous changes with mononuclear cell infiltration can be observed in the control group .However, spindle-shaped fibroblasts proliferation with collagen formation was observed in MSCs-treated wound tissue.The proliferation of fibroblasts with collagen matrix in MSCs-treated wound tissue suggested the formation of granulation tissue and progress of the wound-healing process.Compared with the untreated group, we observed a significant reduction of the wound areas in the MSC-treated group. These results shown that MSCs accelerated the process of wound healing in a rat wound-healing model.

Electrospun polymeric membranes are the most advanced and efficient wound dressing materials compared to other modern bandages such as hydrocolloids, hydrogels, and alginates. These membranes, due to their very high surface area to volume ratio, have extraordinary abilities such as controlled release of drugs, cultivation in the physiological environment of the cells, a very high increase in the reproduction and growth of live cells, etc. All these characteristics have highly extended their applications on different wounds as compared to traditional wound dressing materials.These experiments shown that MSCs promote the wound healing process and help fibroblasts to secrete wound healing- related proteins. Many studies have reported that MSCs secrete a broad spectrum of cytokines and growth factors, particularly vascular endothelial growth factor (VEGF), monocyte hemoattractant protein-1, and hepatocyte growth factor.These growth factors stimulate fibroblasts to proliferate, aid the inflammatory response of wound healing, and accelerate the process of angiogenesis. In the present study, we

demonstrated that MSCs have an effect on the promotion of fibroblast migration and proliferation. The results from the migration assay showed that the number of migrated cells increased in the MSC treated group. These results suggest that MSCs enhance wound healing in diabetic animal models.

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