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Polyhydroxybutyrate-co-hydroxyvalerate structures loaded with adipose stem cells promote skin healing with reduced scarring

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ABSTRACT

Currently available skin substitutes are still associated with a range of problems including poor engraftment resulting from deficient vascularization, and excessive scar formation, among others. Trying to overcome these issues, this work proposes the combination of poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) structures with adipose-derived stem cells (ASCs) to offer biomechanical and biochemical signaling cues necessary to improve wound healing in a full-thickness model. PHBV scaffold maintained the wound moisture and demonstrated enough mechanical properties to withstand wound contraction. Also, exudate and inflammatory cell infiltration enhanced the degradation of the structure, and thus healing progression. After 28 days all the wounds were closed and the PHBV scaffold was completely degraded. The transplanted ASCs were detected in the wound area only at day 7, correlating with an up-regulation of VEGF and bFGF at this time point that consequently led to a significant higher vessel density in the group that received the PHBV loaded with ASCs. Subsequently, the dermis formed in the presence of the PHBV loaded with ASCs possesses a more complex collagen structure. Additionally, an anti-scarring effect was observed in the presence of the PHBV scaffold indicated by a down-regulation of TGF-β1 and α-SMA together with an increase of TGF-β3, when associated with ASCs. These results indicate that although PHBV scaffold was able to guide the wound healing process with reduced scarring, the presence of ASCs was crucial to enhance vascularization and provide a better quality neo-skin. Therefore, we can conclude that PHBV loaded with ASCs possesses the necessary bioactive cues to improve wound healing with reduced scarring.

1. Introduction

Skin wound healing is a complex process that requires a coordinated interplay among cells, growth factors, and extracellular matrix (ECM) proteins [1]. Despite all the research efforts and the commercially available skin substitutes, the regeneration of functional skin remains elusive [2–5]. Besides failed engraftment and poor vascularization, hypertrophic scarring and keloid formation are other possible negative outcomes for skin grafts [6,7]. Scars, in addition to complete loss of tissue function, have psychologically associated issues such as esthetic disfiguration and pain that significantly affect patient's quality of life [8].

Tissue engineering approaches, namely the combination of stem cells with a biomaterial-based matrix, faces the major challenge of introducing the proper cues to induce skin regeneration while avoiding scarring. Adipose-derived stem cells (ASCs) have emerged as attractive players in the improvement of wound healing. It is already known that ASCs actively participate in the different phases of wound healing [9] by secreting different anti-inflammatory cytokines [10], promoting neo-vascularization and re-epithelialization [11–13], and also by regulating fibroblasts'

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phenotype and respective ECM deposition [14]. Additionally, recent works have associated reduced scarring as an effect of ASCs, although the mechanism has not been completely elucidated [15,16]. Interestingly, the need for an approach that avoids cell death and/or migration from the wound site is reinforced in one of these works [15] and has been supported by others that demand the recapitulation of the complex microenvironments of stem cells to maximize their therapeutic potential [17–19]. This can be achieved by tailoring the properties of supporting biomaterials and consequently cells' secretome [20].

Poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV), a natural polymer produced by a range of bacteria, has been explored for skin wound healing [21–27]. PHBV electrospun membranes were shown to influence the re-epithelialization and the rate of healing of mice in full thickness wounds [21,22,25]. While scarring was not directly addressed, Kuppan et al. [25] showed that the mechanical properties of electrospun and solvent cast PHBV matrices influenced the expression of ECM proteins and suggested that electrospun meshes, with higher elasticity, would favor wound healing progress.

Our group has recently developed a PHBV scaffold with a porosity of 82.2% ± 0.5% and an average pore size of 122.4 μm ± 58.1 μm that possesses high water retention capability to maintain wound's moisture [28]. This PHBV scaffold is susceptible to the degradative action of inflammatory enzymes thus supporting new tissue formation while the biomaterial is being degraded. It also presents a stiff character that is expected to better withstand the mechanical stresses that occur during matrix deposition, providing a biomechanical signal which is favorable to an anti-scarring environment. It is also known that ASCs, in addition to the described actions along the healing process, also secrete anti-scarring factors such as TGF-β3. We have therefore hypothesized that the ASC secretome together with PHBV biomechanical features synergize to promote a better wound healing environment by enhancing early vascularization and reducing scarring.

2. Materials and methods

2.1. Animals

A total of 27 Lewis rats, weighing 230–250 g (8 weeks old), were used. Three transgenic rats (Lewis LEW-Tg (eGFP) F455.5/Rrrc; Rat Resource and Research Center, USA) which express enhanced green fluorescent protein (eGFP) were used to obtain endogenously labeled adipose stem cells (ASCs). Twenty-four males, wild-type from the inbred lineage LEWIS/H were used as recipient animals. All experimental protocols were performed in accordance with the Ethical Principles of Animal Experimentation and approved by the Animal Committee of Federal University of Minas Gerais (CEUA/UFMG, protocol 168/2013).

2.2. Preparation of PHBV scaffolds

PHBV scaffolds were prepared as described previously [28]. Briefly, the PHBV polymer (Mw 425.7 KDa, 12% HV content) provided by PHB Industrial, Serrana, Brazil was dissolved in chloroform (Fisher Scientific, UK) at 60 °C under constant agitation to form a homogeneous 2.5% w/v polymer solution. A 3D porous scaffold was produced by freeze-drying. After getting a homogeneous polymer solution, acetic acid (1:1, VWR, UK) was added to obtain an emulsion that was frozen for 48 h at −80 °C and subsequently freeze-dried (Telstar, Spain) for 94 h. Structures were cut into cylinders of 1.2 cm diameter and 2 mm thickness and sterilized by 15 kGy gamma radiation for 30 min prior to cell culture. The surface morphology was examined by scanning electron microscopy (SEM). The scaffolds were coated with 20 nm Au–Pd (80/20 wt.%) using a high resolution sputter coater (208HR, Cressington Company, UK) coupled to a MTM-20 High Resolution Thickness Controller (Cressington Company, UK), and analyzed on a Nova NanoSEM 200 (FEI, The Netherlands) at 5.00 kV.

2.3. Isolation and culture of ASCs

ASCs were isolated from gonadal adipose tissue depots, collected from rats endogenously expressing eGFP. The adipose tissue depots were washed with phosphate-buffered saline (PBS; Sigma, USA) and digested with 0.15% collagenase II (Sigma–Aldrich, USA) at 37 °C for 1 h under agitation. Collagenase activity was then inhibited by the addition of fetal bovine serum (FBS; Gibco, USA) and the digested tissue was centrifuged at 800g for 10 min. Pellet was suspended and plated in culture flasks with DMEM (Gibco, USA), supplemented with 10% FBS, and 1% antibiotic/antimycotic (Invitrogen, USA). Cell cultures were kept in a 5% CO_2 humidified atmosphere at 37 °C for 24 h before medium change, then carried out every 3 days. The mesenchymal population was selected based on its ability to adhere to the culture plate. At 80–90% confluence, cells were detached using 0.25% trypsin–EDTA (Gibco, USA) and replated at a 1:3 ratio. Cells were used at the third passage in all experiments.

2.4. Flow cytometry

ASCs suspensions were incubated with the primary antibodies: mouse anti-rat CD34, CD45, CD54, CD73 and CD90 (1:50, all from Abcam, UK) for 30 min at room temperature. After washing with PBS, cells were incubated with the secondary antibody, Alexa Fluor 555-labeled goat anti-mouse IgG (Molecular Probes, USA), for 30 min at 4 °C, washed again and fixed in 1% formaldehyde (Sigma, USA). Cells incubated only with the secondary antibody were used as control to exclude nonspecific binding. Quantitative analysis was performed using a FACScan argon laser flow cytometer (BD Biosciences, USA). For each sample, 15,000 events were acquired and analyzed using the WinMDI 2.8 software.

2.5. ASC differentiation assay

ASCs were seeded in six well-plates at a density of 10^5 cells per well and allowed to reach confluence (80–90%). Confluent cultures were then maintained in adipogenic [DMEM, 10% FBS, 1% antibiotic/antimycotic, 50 μM indomethacin (Sigma, USA), 0.5 mM isobutylmethylxanthine (Sigma, USA), 0.1 μM dexamethasone (Achè, Brazil), 100UI insulin (Lilly, USA)] or osteogenic [DMEM, 10% FBS, 1% antibiotic/antimycotic, 10^{-8} M dexamethasone (Achè, Brazil), 10 mM de β-glycerophosphate (Sigma, USA), 50 μg/mL ascorbate-2-phosphate (Ecibra, USA)] differentiation medium for 21 days. Media was changed every 3 days.

At the endpoint cells were fixed and stained with Oil Red O (Thermo Scientific, USA) or Von Kossa (Abcam, USA) following the manufacturers' instructions, respectively to assess adipogenic and osteogenic differentiation.

2.6. PHBV construct

To prepare PHBV constructs (PHBV + ASCs), 1×10^6 ASCs were seeded on the PHBV scaffold. Constructs were incubated in a 5% CO_2 humidified incubator at 37 °C for 1 h to allow cell adhesion. After that time, constructs were maintained in DMEM plus 10% FBS and 1% antibiotic/antimycotic for 3 days before *in vivo* implantation.

ID	Title	Pages
327	Polyhydroxybutyrate-co-hydroxyvalerate structures loaded with adipose stem cells promote skin healing with reduced scarring	12