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Research paper

Development and bioevaluation of nanofibers with blood-derived growth factors for dermal wound healing

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ABSTRACT

The aim of our work was to produce a modern nanomaterial with incorporated blood-derived growth factors, produced by electrospinning, applicable in treatment of chronic wounds. Platelet-rich plasma was chosen as a natural source of growth factors. Results showed that platelet-rich plasma stimulates keratinocyte and fibroblast cell growth *in vitro*. Its optimal concentration in growth medium was 2% (v/v) for both types of skin cells, while higher concentrations caused alterations in cell morphology, with reduced cell mobility and proliferation. In the next step hydrophilic nanofibers loaded with platelet-rich plasma were produced from chitosan and poly(ethylene oxide), using electrospinning. The morphology of nanofibers was stable in aqueous conditions for 72 h. It was shown that electrospinning does not adversely affect the biological activity of platelet-rich plasma. The effects of nanofibers with incorporated platelet-rich plasma on cell proliferation, survival, morphology and mobility were examined. Nanofibers limited cell mobility, changed morphology and stimulated cell proliferation. Despite of the small amount of blood-derived growth factors introduced in cell culture via platelet-rich plasma-loaded nanofibers, such nanofibrillar support significantly induced cell proliferation, indicating synergistic effect of nanotopography and incorporated growth factors. The overall results confirm favorable *in vitro* properties of produced nanofibers, indicating their high potential as a nanomaterial suitable for delivery of platelet-rich plasma in wound healing applications.

1. Introduction

Wound healing is a complex physiological process, orchestrated by a variety of biological and mechanical signals in a specific time sequence [1–3]. Tissue injury is followed by several responses of cells associated with wounded site, such as cell adhesion, proliferation, differentiation and migration, synthesis and remodeling of extracellular matrix [1,2,4]. The repair process, initiated immediately after injury, is moderated by the interplay of various growth factors, cytokines and low molecular weight compounds released during platelet degranulation [3].

Growth factors are polypeptides that act as tools for intercellular communication and control of cell fate. They exert their effects locally as regulators of basic cell functions [1,2,5]. Platelets are a well-known natural source of growth factors and are prepared from anticoagulated blood by a separation procedure, which involves a two stage centrifugation. The first one with higher centrifugal force separates buffy coat from red blood cells and plasma, while the second one with low centrifugal force separates platelet-rich plasma (PRP) from remaining red blood cells [6]. Different additive solutions can partially substitute plasma as a storage medium in a final product.

Platelets can be considered as endogenous living depots of a wide range of growth factors. They are concentrated during PRP preparation in a small volume of plasma, therefore providing supraphysiological concentrations of growth factors and cytokines in physiologically relevant ratios [5]. The application of PRP in

Abbreviations: CS, chitosan; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; FBS, fetal bovine serum; HGF, hepatocyte growth factor; IGF, insulin like growth factor; MEM, minimum essential medium; PBS, phosphate buffered saline; PCL, polycaprolactone; PDGF, platelet-derived growth factor; PEO, poly(ethylene oxide); PF4, platelet factor-4; PGA, poly(glycolic acid); PLGA, poly(lactic-co-glycolic acid); PRP, platelet-rich plasma; RMA, relative metabolic activity; SEM, scanning electron microscopy; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.

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therapy is much more effective compared to administration of a single isolated growth factor [5–8]. A wide variety of growth factors can be found in PRP including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), insulin like growth factor (IGF), hepatocyte growth factor (HGF), angiopoietins, platelet factor-4 (PF4), thrombospondin and other proteins such as fibrinogen, fibronectin and vitronectin [4,6,9]. Therapy with PRP has already been proven to be effective in healing of different tissues, such as chronic skin wounds (e.g. diabetic and pressure ulcers) [9–11], tendon and ligament injuries [6,9,11–13] and osteochondral defects [6,9].

Nowadays PRP is usually used as a bolus injection administered into a wounded area [14]. Since it is well known that the local concentration of growth factors in a wound is a critical parameter which affects the healing process, we believe that the biological effectiveness of PRP can be enhanced with its incorporation into a dosage form, which would control its release. Formulation of a scaffold mimicking the structure and functions of natural extracellular matrix (ECM), would also be favorable [15]. Nanofibers, defined as solid fibers with a diameter in the nanometer range and theoretically unlimited length, represent a material which enables the engineering of the aforementioned dosage form or a tissue scaffold [10,16]. Polymer nanofibers mimic the fibrillar elements of natural ECM, which provides biological and physical support for cell attachment, proliferation, migration and differentiation [17]. Therefore, nanofibers offer a great potential for applications in various biomedical fields, especially in tissue regeneration and wound healing [10,16].

The aim of our research was to formulate a nanodelivery system loaded with PRP intended for application in wound healing. Firstly, the effects of PRP on keratinocytes and fibroblasts were assessed and critically evaluated *in vitro*. In the next step biocompatible polymer nanofibers loaded with PRP were produced by electrospinning. Finally, the effect of nanofibrillar support with incorporated PRP on the growth, metabolic activity and morphology of key dermal cells was evaluated to explore the potential synergistic effects of the fiber nanotopography and PRP-derived growth factors on dermal cells, in order to predict their behavior in wound regeneration *in vivo*.

2. Materials and methods

2.1. Materials

Chitosan (CS, low molecular weight) and poly(ethylene oxide) (PEO, Mw 900,000 g/mol) were supplied by Sigma–Aldrich (Germany). The PRP was provided by the Blood Transfusion Centre of Slovenia, Ljubljana, Slovenia. The agarose and cell-culture reagents were also obtained from Sigma–Aldrich (Germany), unless otherwise indicated.

2.2. PRP preparation and handling

Buffy coat was prepared from whole blood of a random blood donor according to a standard blood bank procedure. Briefly, 450 ml of citrated whole blood collected in a Top&Bottom bag system (Fresenius Kabi, Germany) was centrifuged at 20 °C and 58,000 rpm (Cryofuge 6000i, Hereaus, USA). Packed red blood cells (RBCs) and plasma were transferred to satellite bags by using the Compomat G5 automatic seperator (Fresenius Kabi, Germany) with approx. 50 ml of buffy coat remaining in the primary bag. Five such buffy coats of blood donors with the same ABO group were pooled together by using 250 ml of InterSol additive solution (Fenwal Inc., Il.). Pooled cells were first centrifuged (1 min at approx. 90g, with slow decantation during centrifugation), and then the transfer of PRP to a final bag was carried out through a leukodepletion filter on an Orbisac machine (Caridian, USA). The final product, containing about 1×10^9 platelets and less than 3,000 leukocytes per ml was finally divided in aliquots that were frozen at −80 °C and thawed just before use. The freezing–thawing procedure resulted in lysis of platelets and a consequent release of intracellular growth factors. Qualitative and quantitative analysis of PRP in terms of individual growth factor composition was not determined, as this would be beyond the scope of the study.

The amount of dry weight of PRP was determined following lyophilization. Briefly, 1 ml of frozen PRP was lyophilized at room temperature and 0.09 mbar, for 24 h. The dry weight of PRP was determined gravimetrically from the weight difference of container prior and after lyophilization. The experiment was carried out in five parallels.

2.3. Preparation of polymer solutions

The blended polymer solution was prepared by mixing CS (2%, w/w) and PEO (3%, w/w) solutions in 3% (v/v) acetic acid in water, to achieve the final 50:50 (w/w) ratio of polymers. Triton® X-100 (0.3%, w/v) and DMSO (10%, v/v) were added to the CS/PEO blended solution which was then stirred at room temperature for another 5 h using a magnetic stirrer.

CS/PEO solutions with PRP were prepared by addition of 0.25 or 0.5 ml of thawed PRP to 4.75 or 4.5 ml of 2.9% (w/w) CS/PEO solution (ratio of polymers 50:50 (w/w)), supplemented with Triton® X-100 (0.3%, w/v) and DMSO (10%, v/v). The solutions were prepared freshly, just before electrospinning.

2.4. Electrospinning

The polymer solution was placed in a plastic 20 ml syringe fitted with a metal needle (the inner diameter of 0.8 mm). The syringe was mounted in a pump (model R-99E, Razel™) that maintained a steady flow rate. The high voltage was supplied by a voltage generator (model HVG-P60-R-EU, Linari Engineering s.rl. Italy) connected to the needle. A planar stand covered with aluminum foil was used as a collector. The electrospinning was performed in a sealed chamber, which enabled controlled electrospinning environment i.e. relative humidity of 34% and temperature of 27 °C. The process conditions used are shown in Table 1.

For their *in vitro* evaluation in cell cultures the nanofibers were electrospun on glass cover slips, which fit into the wells of cell culture plates used. To keep the constant amount of nanofibers deposited on glass coverslips, the electrospinning was always performed for 15 min under conditions shown in Table 1.

2.5. Morphology of nanofibers

The morphology of produced nanofibers was characterized by scanning electron microscopy (SEM). The samples of nanofibers were fixed onto metallic studs with double-sided conductive tape (diameter 12 mm, Oxford instruments, Oxon, UK) and analyzed using a Supra 35 VP (Carl Zeiss, Oberkochen, Germany) scanning electron microscope with an acceleration voltage of 1 kV and a secondary-electron detector. The SEM images were used to determine the nanofiber morphology and their average diameter, which was calculated based on measurements of thirty randomly chosen nanofibers, using ImageJ 1.44p software (NIH, USA).

To determine their stability in aqueous medium the nanofibers were soaked in PBS for 72 h, then the medium was discarded, nanofibers gently washed with water, air dried and analyzed with SEM, according to the previously described procedure.