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Calcium alginate particles for the combined delivery of platelet lysate and vancomycin hydrochloride in chronic skin ulcers

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ABSTRACT

The aim of the present work was the development of a powder formulation for the combined delivery of platelet lysate and of a model antibiotic drug, vancomycin hydrochloride (VCM), in chronic skin ulcers. Calcium alginate particles were prepared by freeze-drying beads obtained by ionic gelation method. The experimental conditions adopted permitted the complete loading of VCM and of PDGF AB, the growth factor chosen as representative of those contained in PL.

Such particles where able to absorb PBS (mimicking wound exudate), to form a gel and to modulate the release of VCM and of PDGF AB. They are characterized by enhancement properties of human fibroblast proliferation due to PL presence. In particular, PL, when loaded in alginate particles, was able not only to increase the number of viable cells, but also the number of cells in proliferative phase. Such properties were comparable to those of fresh PL indicating the capability of calcium alginate particles to load PL bioactive substances without altering their activity. The formulation developed is characterized by an easier and a less painful administration with respect to traditional gauzes and semisolid preparations and permits the loading in the same dosage form of active substances of different nature avoiding eventual incompatibility problems.

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1. Introduction

Chronic wounds are classified as wounds that do not heal in 12 weeks, due to repeated tissue insults or to endogenous factors such as underlying pathologies (Boateng et al., 2008). Among these there are diabetes, tumours, immunodeficiency, metabolic and connective tissue disorders. Chronic wounds include decubitis ulcers (bedsores or pressure sores) or leg ulcers (venous, ischaemic or of traumatic origin).

In the last decades the progress in research has led to define the complexity of the events involved in tissue regeneration. The wound healing is a tissue regenerating process mediated by different interdependent matrix and cellular events that overlap to generate the reconstruction of mesenchymal cells layer, cellular proliferation and extracellular formation in order to re-establish the integrity of damage tissue (Crovetti et al., 2004).

A chronic wound fails to heal because the orderly sequence of events is disrupted at one or more of the stages of wound healing.

The main cellular activities involved in tissue repairing process are mediated by a pool of growth factors (GFs), cytokines and proteins from the serum and degranulating platelets. Although the role of all the GFs involved in tissue regeneration is only partially elucidated, the potential benefits of many of them have been demonstrated. For example, platelet-derived growth factor (PDGF) is a powerful mitogen for connective tissue cells, transforming growth factor-β (TGF-β) stimulates re-epithelization, granulation tissue formation, angiogenesis, fibroblast proliferation, myofibroblast differentiation and matrix deposition; insulin like growth factor (IGF-I) promotes mitogenesis; vascular endothelial growth factor (VEGF) induces endothelial cell proliferation and migration, thus initiating the angiogenic response (Anitua et al., 2007; Denis et al., 2012; Werner and Grose, 2003).

In the last decade, the therapeutic use of hemoderivatives derived from platelets has been suggested in wound treatment (Borzini and Mazzucco, 2005; Crovetti et al., 2004; Dohan Ehrenfest and Rasmusson, 2009; Mazzucco et al., 2004; Ogino et al., 2006). Platelets are specialized secretory cells that release, in response to activation, a large number of biologically active substances from intracellular alpha granules and in particular GFs involved in the healing process. Among platelet derivatives, platelet lysate (PL) has been proved to be effective in wound healing (Ranzato et al., 2008).

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In previous works of ours PL lysate was successfully loaded in gel and sponge-like dressings intended for the treatment of mucosal, corneal and skin lesions. Such formulations were capable to enhance *in vitro* proliferation of fibroblasts and corneal (RCE) cells (Del Fante et al., 2011; Sandri et al., 2011a, 2011b, 2012; Rossi et al., 2013a; Caramella et al., 2013).

The presence of bacterial infections can inhibit or negatively influence wound healing. Since wound environment is a good culture medium for microorganism proliferation, wound colonization is frequent and generally involves different microorganisms that can lead to infections. These, besides resulting in prolonged healing times, can cause cell inflammation (cellulitis), followed by bacteremia and septicemia that can be fatal (Boateng et al., 2008). For this reason the treatment of chronic wounds provides the local or systemic administration of anti-infective drugs.

Given these premises, the aim of the present work was the development of a topical powder for the combined delivery of PL and of an antimicrobial drug, vancomycin HCl (VCM), in chronic wounds. Such a powder was obtained by dropping an alginate solution into a $CaCl_2$ solution to form beads for ionic gelation, and by the subsequent freeze-drying of beads. Freeze-dried beads (calcium alginate particles) loaded with PL and VCM were prepared and mixed together to obtain a powder containing the hemoderivative and the anti-infective drug. Such a powder, once applied to the wound, should be able to absorb wound exudate, hydrating itself to form a gel, and to release the loaded bioactive substances.

The work was divided in two phases. The first one was focused on detecting the optimal experimental conditions to form calcium alginate particles. Particles size and hydration properties in PBS (phosphate buffer saline), medium mimicking the wound exudate, were evaluated.

Once the best experimental conditions were found, in the second phase of the work, calcium alginate particles loaded with PL and VCM were prepared. They were characterized for encapsulation efficiency and/or loading capacity and *in vitro* release properties.

Calcium alginate particles loaded with PL were subjected to *in vitro* proliferation and wound healing tests on fibroblasts in order to investigate the influence of the excipients and of preparation conditions on PL activity. Fresh PL was considered as reference.

2. Materials and methods

2.1. Materials

The following materials were used: acetonitrile (Carlo Erba, Milan, I); alginic acid sodium salt, from brown algae, low viscosity grade (~250 cps) (Sigma Chimica, Milan, I); Alginic acid sodium salt, from brown algae, medium viscosity grade (~2000 cps) (Sigma Chimica, Milan, I); 5-bromo-2′-deoxyuridine (BrdU) (Sigma Aldrich, Milan, I); $CaCl_2$ (Sigma Chimica, Milan, I); Dulbecco's Modified Eagles Medium (DMEM, Lonza, BioWhittaker, B); Dulbecco's Phosphate Buffer Solution (Sigma Aldrich, Milan, I); anti-mouse IgG-FITC antibody (Sigma Aldrich, Milan, I); Hank's balance salt solution (HBSS) (Sigma Aldrich, Milan, I); Hoechst 33258 (Sigma Aldrich, Milan, I); inactivated foetal calf bovine serum (Euroclone, Pero, I); mouse anti-bromo-deoxyuridine antibody (anti-BrdU antibody) (Amersham Bioscience, USA); Mowiol (Sigma Aldrich, Milan, I); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, Milan, I); $NaH_2PO_4{\cdot}H_2O$ (Carlo Erba, Milan I); $Na_2HPO_4{\cdot}H_2O$ (Carlo Erba, Milan, I); NaCl (Carlo Erba, Milan, I); Penicillin streptomycin 100× (pen/strep)/amphoteric 100× (Sigma Aldrich, Milan, I); PTA-blocking solution (PBS/Tween20/Albumin) (Sigma Aldrich, Milan, I); trypan blue solution (Biological Industries, Beit-Haemek, IL); trypsin–EDTA solution (Sigma Aldrich, Milan, I); vancomycin hydrochloride USP (Pharmatex Italia s.r.l., Milan, I).

Platelet lysate was supplied by Immunohaematology and Transfusion Service and Cell Therapy Unit of Fondazione IRCCS, S. Matteo, Pavia, I. A pooled sample prepared from platelet rich plasma (containing 500×10^3 platelets/μl) obtained from eight different blood donors was used.

2.2. Preparation of unloaded freeze-dried beads (calcium alginate particles)

Calcium alginate beads were prepared by ionic gelation method and different experimental conditions were considered: alginate viscosity grade, $CaCl_2$ concentration, rest time of beads in $CaCl_2$ solution and needle size. In particular, 1 ml of sodium alginate (SA) solution, low (LV) and medium (MV) viscosity grade, at different concentrations (0.5%, 0.75%, 1% and 2%, w/w) were dropped with two types of syringe needles (30 G: 0.30 ⌀ × 12.7 mm – 32 G: 0.26 Ø × 4 mm) into 30 ml of $CaCl_2$ solution (0.5–1%, w/w). The rest time in the calcium chloride solution was also investigated (10 and 30 min). Beads were separated by vacuum filtration and freeze-dried overnight (Heto DRY WINNER®, Analitica De Mori, Milan, I) to form calcium alginate particles.

2.3. Characterization of calcium alginate particles

2.3.1. Size assessment

Size of calcium alginate particles was evaluated by means of optical microscope (Leica DMI 3000B), equipped with internal camera and PC Leica Application Suite EZ V1.8.1 program (Leica Microequipments, Milan, Italy). Six particles were analyzed for each sample.

2.3.2. Assessment of hydration properties

The hydration properties of calcium alginate particles were evaluated by means of modified Enslin apparatus, using PBS (phosphate buffer saline, $NaH_2PO_4{\cdot}H_2O$ 0.036%, w/w, $Na_2HPO_4{\cdot}H_2O$ 0.137%, w/w, NaCl 0.85%, w/w) as medium to mimic wound exudate. Three replicates were performed for each sample. As an example, Fig. 1 reports photographs of particles placed in contact with PBS for different times.

2.3.3. Assessment of cell proliferation properties

NHDF fibroblasts (juvenile fibroblast from foreskin) (Promocell GmbH, Heidelberg, G) from 6th to 16th passage were used.

Cells were cultured in a polystyrene flask (Greiner bio-one, PBI International, Milan, I) with 13–15 ml of complete culture medium (CM), consisting of Dulbecco's modified Eagles medium with 4.5 g/l glucose and L-glutamine supplemented with 1% (v/v) penicillin streptomycin 100x (pen/strep), 1% (v/v) amphoteric 100× and 10% (v/v) inactivated foetal calf bovine serum. Cells were maintained in incubator (Shellab® Sheldon® Manufacturing Inc., Oregon, USA) at 37 °C with 95% air and 5% CO_2 atmosphere. All the operations required for cell culture were carried out in a vertical laminar air flow hood (Ergosafe Space 2, PBI International, Milan, I). After the cells had reach 80–90% of confluence in the flask (one week) a trypsinization was performed: at first the monolayer was washed with Dulbecco's Phosphate Buffer Solution in order to remove bivalent cations that could inactivate trypsin, then 3 ml of 0.25% (v/v) trypsin-EDTA solution were left in contact with the monolayer for 5 min. After that time, the cell layer was harvested with 7 ml of the complete medium to stop the proteolytic activity of trypsin and to facilitate the detachment of cells. Afterwards, cells suspension was centrifuged (TC6, Sorvall Products, Newtown, USA) at 112 g for 10 min. The supernatant was eliminated and then the cells were re-suspended in 6 ml medium without serum (M, w/s). The amount of cells in suspension was determined in a counting chamber (Hycor

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