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rhEGF-LOADED PLGA-ALGINATE MICROSPHERES ENHANCE THE HEALING OF FULL-THICKNESS EXCISIONAL WOUNDS IN DIABETISED WISTAR RATS

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ABSTRACT

Diabetic foot ulcers (DFUs) represent a major clinical challenge in the ageing population. To address this problem, rhEGF-loaded Poly-Lactic-co-Glycolic-Acid (PLGA)-Alginate microspheres (MS) were prepared by a modified w/o/w-double-emulsion/solvent evaporation method. Different formulations were evaluated with the aim of optimising MSs properties by adding NaCl to the surfactant solution and/or the solvent removal phase and adding alginate as a second polymer. The characterization of the developed MS showed that alginate incorporation increased the encapsulation efficiency (EE) and NaCl besides increasing the EE also became the particle surface smooth and regular. Once the MS were optimised, the target loading of rhEGF was increased to 1% (PLGA-Alginate MS), and particles were sterilised by gamma radiation to provide the correct dosage for in vivo studies. *In vitro* cell culture assays demonstrated that neither the microencapsulation nor the sterilisation process affected rhEGF bioactivity or rhEGF wound contraction. Finally, the MS were evaluated *in vivo* for treatment of the full-thickness wound model in diabetised Wistar rats. rhEGF MS treated animals showed a statistically significant decrease of the wound area by days 7 and 11, a complete re-epithelisation by day 11 and an earlier resolution of the inflammatory process. Overall, these findings demonstrate the promising potential of rhEGF-loaded MS (PLGA-Alginate MS) to promote faster and more effective wound healing, and suggest its possible application in DFU treatment.

Keywords

Poly-Lactic-co-Glycolic-Acid (PLGA), Alginate, recombinant human epidermal growth factor (rhEGF), wound healing, tissue engineering

1. Introduction

Diabetic foot ulcers (DFUs), one of the most common complications in patients with diabetes, have become a problem in health care systems all over the world. The current increment in DFUs associated risk factors such as, ageing population, smoking and obesity can complicate and slow down the healing process (Loots, et al. 2002). The etiology of DFU is related to the damage of blood vessels, nerves and immune system modulation, caused by the long-term hyperglycaemia suffered by diabetic patients (Cevc and Vierl 2010; Falanga 2004). The ulcers are characterised by extracellular matrix (ECM) synthesis and a remodelling disorder due to dermal fibroblast alteration, phenotypic alteration of the matrix metalloproteinases, re-epithelisation disturbance and a persistent inflammatory response (Cook, et al. 2000).

The administration of growth factors, involved in wound healing, has shown limited success. For example, Regranex® (Becaplermin) is a gel containing human recombinant platelet-derived growth factor (rhPDGF) that is approved by the U.S. Food and Drug Administration (FDA). It requires daily application for neuropathic wound healing (Goldman 2004). A postmarket epidemiologic study revised by the FDA demonstrated that, patients who had been prescribed Regranex® three or more times had a five times increased risk of death from cancer. Nevertheless, the duration of follow-up of patients in this study was not long enough to detect new cancers. As a result, the potential risk of using Regranex® should be weighed against the benefit for each individual patient (Update of Safety Review, June 1, 2010). There are also two commercialised treatments based on rhEGF administration. Rengen-D 150TM is a gel containing 150 µg/g of Recombinant Human Epidermal Growth Factor (rhEGF) that was commercialised in India for the treatment of grade I or II diabetic ulcers. It requires twice daily administration, with an average treatment period of 6 weeks (Mohan 2007). Heberprot®,

a lyophilised formulation containing 75 µg of rhEGF is administered 3 times weekly by intralesional injections. It is marketed in Algeria, Argentina, Colombia, Cuba, the Dominican Republic and Venezuela. A pilot study carried out in 20 diabetic patients demonstrated Herberprot® as a feasible and safe treatment to promote the healing of chronic wounds in patients with full thickness ulcers (Fernandez-Montequin, et al. 2009). However, rhEGF´s short half life requires a continuous exposure (at least 6-12 hours) to enhance the mitogenic effect on epithelial (Hardwicke, et al. 2008; Ulubayram, et al. 2001). Therefore, in order to achieve a significant therapeutic effect, it is necessary to optimise the administration of growth factors such as rhEGF, in terms of dose, delivery system and safety.

With the aim of overcoming the previously described limitations, we developed rhEGF polymeric microspheres using a w/o/w double-emulsion solvent evaporation method. The microencapsulation process is used to provide a sustained and controlled release of rhEGF, avoid rhEGF inhibition by wound proteases, elude frequent drug administration and prolong treatment effectiveness (Chu, et al. 2010; Dong, et al. 2008). rhEGF was encapsulated using PLGA (Poly D,L-Lactic-co-Glycolic Acid) and alginate. On the one hand, the PLGA polymer has the advantage of being biocompatible and biodegrades in a controlled manner (Jain 2000).On the other hand, alginate, a natural polysaccharide, has a chemical structure similar to ECM (extracellular matrix) components. Moreover, according to Jay, et al. 2008, the use of alginate could increase de encapsulation efficiency in cases that the amount of the encapsulating therapeutic proteins is scarce (Jay, et al. 2008).

In this study, we optimised the preparation of rhEGF-loaded PLGA-Alginate microspheres (MS). Furthermore, *in vitro* experiments were undertaken to determinate the susceptibility of fibroblasts to proliferate being in contact with encapsulated rhEGF,

the bioactivity of encapsulated rhEGF and the migration and proliferation of fibroblasts in the presence of the encapsulated growth factor. Finally, *in vivo* studies were carried out to demonstrated whether intralesional administration of a single dose of 75 µg of rhEGF microspheres accelerated wound healing in terms of wound contraction, epidermal regeneration and inflammatory stage recovery.

2. Materials and methods

2.1. Microsphere preparation

Microspheres containing rhEGF were prepared using a w/o/w double emulsion/solvent evaporation method. Briefly, 2 ml of 5% (w/v) of PLGA Resomer RG503 [MW40,600; viscosity 0.41 dl/g in chloroform 0.1%, 25ºC, with a copolymer ratio of 50:50 lactic/glycolic (%)] (Evonic industries, Germany) in dichloromethane:acetone (3:1) solution was emulsified for 15 s at 50 W (Branson® 250 sonifier, CT, USA) with 0.2 ml of an internal aqueous phase (in MilliQ water) containing 100 µg rhEGF (Center for Genetic Engineering and Biotechnology, Cuba), 5 mg of Human Serum Albumin (HSA) (used as a stabiliser of the rhEGF) and 0.5 mg of polyethylene glycol 400 (PEG400). The resulting w_1/o emulsion was poured into a surfactant solution, containing 15 ml of 5% (w/v) polyvinyl alcohol (PVA) in miliQ water and mixed with a paddle stirrer (Biocote® Stirrer SS20) for 60 s to perform the double emulsion ($w_1/o/w_2$). Finally, an organic solvent removal phase comprising 400 ml of miliQ water was added and stirred for 30 min. The MS were then collected by filtration and lyophilised. This formulation was called MS1. The target loading of rhEGF in MS was 0,1% (w/w).

Different changes were included in the preparation protocol described before in order to increase the encapsulation efficiency (EE). Table 1 summarised the 4 different formulations developed in the MS optimisation process. The two main changes were: (i) NaCl incorporation into the surfactant phase (formulation MS2) as well as in the solvent

removal phase (MS3 and MS4). (ii) addition of a second biopolymer, sodium alginate MVG [MW 200,000–300,000 g/mol, medium viscosity, monomer ratio 60:40 guluronic/mannuronic (%)] (Pronova UP, NovaMatrix FMC BioPolymer, Sandvika, Norway), and $CaCl_2$ to enable the gelation of alginate (MS4). After the optimisation of microspheres, the target loading of rhEGF was increased to 1% (PLGA-Alginate MS) to provide an appropriate dosage for *in vivo* studies.

2.2. Gamma-irradiation of rhEGF-loaded microspheres

rhEGF-loaded MS (PLGA-Alginate MS) were sterilised by γ-irradiation (PLGA-Alginate MS-γ) for the *in vivo* studies. Microspheres were placed in 5 ml glass vials and covered with dry ice to ensure a low temperature. A dose of 25 kGy from ^{60}Co, as the radiation source (Aragogamma S.A., Barcelona, Spain), was used to ensure effective sterilisation, in accordance with European Pharmacopeia recommendations (Fernández-Carballido, et al. 2006). Then, the influence of the sterilisation process on the properties of the formulation was studied.

2.3. Microsphere characterisation

Size distribution was measured by laser diffractometry with a Coulter Counter® LS130 particle size analyser. Surface appearance and sphere morphology was determined by scanning electron microscopy (SEM; Jeol® JSM-35 CF). The zeta potential was estimated by means of photon correlation by Malvern® Zetasizer Nano ZS. Each assay was performed in triplicate.

2.4. Determination of rhEGF encapsulation efficiency

1 milligram of MS was dissolved in 0.4 ml of dimethyl sulphoxide (DMSO), vortexed for 5 minutes and mixed with 0.6 ml of a solution of Dulbecco's Phosphate-Buffered

Saline (DPBS) containing 0.05% (v/v) Tween 20 and 0.1% (w/v) Bovine Serum Albumin (BSA).

The amount of rhEGF loaded was estimated by a commercially available Sandwich Enzyme-Linked Immunosorbent assay kit for human EGF (human EGF ELISA development kit, Peprotech). The assay was performed according to the manufacturer's instructions.

Encapsulation efficiency was calculated as the percentage of the encapsulated rhEGF, referred to the total initial amount of rhEGF used for the microsphere preparation. All the tests were performed in triplicate, and the results reported as the means ± S.D.

2.5. *In vitro* release studies

The rhEGF *in vitro* release profile was determined by incubating 5 mg of MS with 1 ml of 0.02 M phosphate-buffered saline (PBS) (pH 7.4) at 37 ± 0.5 ºC under orbital rotation at 25 rpm. The release medium was removed by centrifugation and replaced by the same quantity of PBS at predetermined intervals (Gutierro, et al. 2002). Samples were taken at 30 min., 4, 8 and 10 h. Afterwards, once a day during the first week, and once a week up to 41 days. The amount of rhEGF in the release medium was assayed by ELISA using the protocol described in section 2.4. The rhEGF release profile was only studied in the formulations with the highest EE (MS3 and MS4) and with the highest rhEGF target loading (PLGA-Alginate MS and PLGA-Alginate MS-γ) (n=3).

2.6. *In vitro* cell culture studies

2.6.1. Cell culture

Balb/c 3T3 A31 fibroblasts (ATCC, Manassas, USA) were cultured in a complete culture medium prepared with DMEM (ATCC, Manassas, USA) and supplemented

with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) penicillin-streptomycin. Cells were incubated at 37ºC and 5% CO_2 and were passaged out every 2-3 days.

2.6.2. Bioactivity assay for rhEGF

Balb/C 3T3 cells were seeded in 96-well plates at 4500 cells/well in complete culture medium for 8 hours (n=4). Medium was replaced with 100 µl assay medium containing 0.2% calf serum and incubated overnight. Then, serial dilutions from 2000 pg/ml to 2 pg/ml of rhEGF were made in the assay medium and 100 µl of each dilution was added. The following groups were assayed (n=4): (i) rhEGF obtained from the *in vitro* release studies of PLGA-Alginate MS in 0.2% FCS-supplemented DMEM, (ii) rhEGF obtained from 15 day long *in vitro* release studies of PLGA-Alginate MS-γ in 0.2% FCS-supplemented DMEM and (iii) free rhEGF in 0.2% FCS-supplemented DMEM. Then cells were cultured for 48 hours (Jimenez Hamann, et al. 2005). The bioactivity of the samples was evaluated by measuring cell proliferation adding 10 µl of CCK-8 (Cell Counting Kit-8 for quantisation of viable cell number in proliferation and cytotoxicity assays, Sigma-Aldrich, Saint Louise) to each well. The absorbance was read at 450 nm and at 650 nm as the reference wavelength after 4 hours of incubation. The absorbance was directly proportional to the number of living cells in culture. The results are given as 50% effective dose (ED_{50}): the dose that produces the desired effect in 50 per cent of a population.

2.6.3. In vitro migration assay

Collagen type I coated 6-well plates were seeded with 10^6 Balb/C 3T3 cells/ml in complete medium and cultured at 37ºC for 24 h to form a confluent monolayer. Gaps, of approximately 0.9 mm in width, were created by scratching the plates with a sterile pipette tip (Walter, et al. 2010). The medium was immediately removed and replaced

with (i) 15 ng/ml of free rhEGF in serum-free DMEM, (ii) 15 ng/mL rhEGF obtained from 15 day long *in vitro* release studies (PLGA-Alginate MS) in serum-free supplemented DMEM and (iii) fresh serum-free medium. The width average of the gaps was calculated from the images taken by a microscope at three different sites from each well using ImageJ® software. Photographs were taken at 0, 5, 18 and 24 h after wound creation. Moreover, cell cultures were recorded during 24 h by microscope (Biostation IM-Q, Nikon) in order to observe cell migration and proliferation.

2.7. *In vivo* studies

2.7.1. Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of the Basque Country (Procedure number: CEBA/202/2011/HERNANDEZ MARTIN). Rats were housed in individual cages on a 12 h light-dark cycle with free access to standard rodent chow and water. For *in vivo* experiments, 50 male Wistar rats of 225-250 g were used. Animals were fasted for 8 h and diabetes was induced by a single i.p. injection of sterile 0.5% (w/v) streptozocin (STZ) (Sigma, ST. Louis, MO) dissolved in sodium citrate buffer (0.1 mol/L, pH 4.5) at a dose of 55 mg/kg. The diabetic state was confirmed 72 h after STZ injections when blood glucose levels exceeded 300 mg/dl (Li, et al. 2008).

2.7.2. Wound induction and experimental animal groups

2 wounds of 1 cm in diameter at each side of the midline, were created on the raced dorsum of 1 week diabetised rats (anesthetised by isoflurane inhalation). Full-thickness wounds extending through the *panniculus carnosus* were excised using a sterile surgical blade G21. A donut-shaped silicone splint 1.5 cm in diameter was sutured around the

wound with a 4-0 nylon suture (Aragó, Barcelona) preventing wound contraction, the major healing process in rodents, and allowing healing by re-epithelisation and granulation tissue formation (the manner in which human skin heals) (Michaels, et al. 2007). Animals were divided into the following 5 groups (n=12): (i) untreated control, (ii) vehicle control (0.9% saline and 0.5% carboxymethylcellulose buffer), (iii) empty MS control, (iv) 75 µg of free rhEGF and (v) 75 µg of rhEGF MS (PLGA-Alginate MS) group. MS previously resuspended in 0.5 ml vehicle and vehicle treatments (0.5 ml) were administered by a unique intralesional injection with a 23 G needle and deepening it downward into the wound bottom in circles and centripetally ensuring a uniform distribution. Free rhEGF resuspended in 0.5 ml vehicle was injected in the wound twice a week. After the treatment administration, wounds were covered with one layer of petrolatum gauze (Tegraderm® 3M) and two layers of sterilised adhesive. A brief description of the study protocol is shown in Table 2.

2.7.3. Serial wound analysis

Blood glucose levels were measured 7 and 3 days before wounding and at days 0, 5, 9, 14 and 17 post-wounding. Blood samples were obtained from the tail vein and glycaemia was calculated by a blood glucose test meter (Accu-Chek® Sensor, Roche, Germany). Body weight was measured using an electronic balance 3 days before wounding and at days 0, 5, 9, 14 and 17 after wounding.

Wound closure was determined by measuring the wound area (px^2) on surgery day and on days 4, 7, 11, 14 and 17 after wounding by tracing the wound margin using ImageJ® software. Wound closure was calculated as a per cent area of the original wound. A wound was considered completely closed when the wound area was equal to zero (Galiano and Michaels 2004).

2.7.4. Histological analysis

After 7, 11 and 17 days, animals were euthanised by CO_2 inhalation. The wounds were excised in 5 µm thick layers under the fascia, including a 5 mm margin of unwounded skin, bisected and fixed in 10% formalin for 24 hours. The samples were processed by H&E staining for morphological observations (Li, et al. 2008; Ulubayram, et al. 2001). Re-epithelisation grade was measured according to Sinha and Gallagher 2003 established criteria (Sinha and Gallagher 2003). Results were given in a semiquantitative manner with a score from 0 to 4. 0: re-epithelisation at the edge of the wound, 1: re-epithelisation covering less than half of the wound, 3: re-epithelisation covering more than half of the wound, 3: re-epithelisation covering the entire wound, irregular thickness and 4: re-epithelisation covering the entire wound, normal thickness. The scale described by Cotran et al. 2000 was used to evaluate resolution phases of inflammatory recovery (Cotran, et al. 2000). 1: acute inflammation (formation of fibrin clot and pyogenic membrane; migration of leucocytes and polynuclear neutrophils), 2: predominance of diffuse acute inflammation (predominance of granulation tissue and pyogenic membrane; vascular neogenesis), 3: predominance of chronic inflammation (fibroblast proliferation), 4: resolution and healing (reduction or disappearance of chronic inflammation although occasional round cells may persist).

2.10. Statistical analyses

Data are presented as the mean ± standard deviation. Statistical significance was accepted at $p < 0.05$. Differences in the EE and the release profile before and after gamma sterilization were analyzed by Student's t-test. For multiple group comparisons, one-way ANOVA and post-hoc tests were used. The Bonferroni or Tamhane post-hoc

test was applied based on to the result of the Levene test of homogeneity of variances. All statistical analyses were performed with SPSS 20.0 (SPSS, Inc., Chicago, IL).

3. Results

3.1. Microsphere optimisation

Optimal microencapsulation process was set up by the determination of physicochemical properties of 0.1% rhEGF loaded MS (MS1 to MS4). All MS displayed a similar size and zeta potential (Figure 1A). The addition of NaCl to the surfactant solution and the solvent removal phase increased the EE of MS3 up to 67.53 ± 9.06 % ($p<0.05$ compared with MS1 and MS2), while alginate incorporation led to an even higher EE of 88.11 ± 1.51 % for MS4 formulation ($p<0.05$ compared with MS1, MS2, MS3).

As shown by SEM images (Figure 1B) MS1s were very porous. To reduce the porosity, NaCl introduction into the surfactant solution (MS2) reduced the porosity and the pore size although not completely eliminated. The incorporation of NaCl in the surfactant solution and solvent removal phase became the surface of the MS smooth and regular without pores. No differences in size or morphology were found between the MS that have alginate (MS4) and those without it (MS3).

rhEGF release profile was studied in the formulations with higher EE values (MS3 and MS4). Both formulations present a similar release profile with an initial release (burst release) associated with the surface associated peptide (SAP) of 3.62 ± 0.52% for MS3 and 4.85 ± 0.17% for MS4, followed by a sustained release phase from day 1 up to 41 days (Figure 1C).

Further studies were performed on MS4 formulation based on its higher EE.

3.2. Study of the effect of increasing the rhEGF load and the sterilisation process on MS properties

Particle size, zeta potential values and MS morphology of 1% rhEGF MS, before and after γ-sterilisation, were similar to the 0.1% loaded formulations (MS4) (Figure 1A vs. 2A). In addition, ELISA assays demonstrated that the EE was reduced when the rhEGF loading increased (68.82 ± 1.50%) (Figure 1B vs 2B). I*n vitro* release profiles show an increment on SAP from 4.85 ± 0.17% for MS4 to 24.55 ± 1.65% for 1% rhEGF MS (Figure 1C and 2C). After the γ-sterilisation of the 1% loaded MS, the EE decreased even more (60.01 ± 1.10%) (Figure 2A), being this difference statistically significant ($p<0.05$). In accordance with these results, γ-irradiated MS showed a statistically significant decrease in the burst effect ($p<0.05$), and the differences observed in the release profile were also statistically significant ($p<0.05$) (Figure 2C).

3.3. *In vitro* cell culture studies

3.3.1. Bioactivity assay for rhEGF

The effects of microencapsulation and γ-sterilisation on rhEGF bioactivity were quantified in the bioactivity assay. To assess the activity of the growth factor, the ED_{50} of rhEGF was quantified. A lower ED_{50} value reflects a greater effectiveness of a drug. The biological activity of rhEGF was not significantly affected by the microencapsulation or sterilisation processes (free rhEGF ED_{50} 66.95 ± 10.67 pg/ml, rhEGF obtained from PLGA-Alginate MS ED_{50} 54.89 ± 8.58 pg/ml and rhEGF obtained from PLGA-Alginate MS-γ ED_{50} 60.77 ± 11.75 pg/ml) ($p < 0.05$) (Figure 3).

3.3.2. In vitro migration assay

Balb/c 3T3 fibroblasts closed the wound from the scratch assay in the presence of 15 ng/ml of rhEGF, faster than the control group (Figure 4). At 5 h, the extent of wound closure was 40.64 ± 6.57% for free rhEGF, 40.47 ± 7.06% for rhEGF obtained from the release tests (PLGA-Alginate MS) and 7.00 ± 3.00% for the serum-free medium control group. For both treated groups, free rhEGF and rhEGF obtained from the release tests (PLGA-Alginate MS), the differences with the control group reached statistical significance ($p<0.05$). Complete wound closure of the scratches was achieved in free rhEGF group and rhEGF obtained from the release tests (PLGA-Alginate MS) at 18 h and 24 h, compared to the increased wound area of the serum-free medium control group. rhEGF-treated groups did not change cell morphology. In contrast, in the serum-free medium control group, the elongated and fusiform morphology began to disappear by 5 h. I*n vitro* migration assay shows that cells from the free rhEGF and rhEGF obtained from the release test groups migrated and proliferated, closing the wound completely after 24 h. In the serum-free medium control group, cells did not close the wound thoroughly at 24 h.

3.4. *In vivo* studies

3.4.1. Blood glucose level and body weight

Hyperglycaemia was confirmed 72 hours after streptozocin administration, and the wound induction was performed one week after the STZ treatment. The blood glucose level exceeded 300 mg/dL in all groups during the experiment. Mice showed no adverse reaction to the different treatments through the 17th day, with no significant differences in body weight in any of the experimental groups.

3.4.2. Wound closure

The skin rapidly contracted to cover the wound. 4 days after wounding, the contraction in the rhEGF MS group was significantly different from the free rhEGF group (25.44 ± 11.15% and 36.43 ± 5.45%, respectively, $p<0.01$) (Figure 5A). Therefore, the rhEGF treated groups (Free rhEGF and rhEGF MS) presented a more reduced wound area (one-way ANOVA, $p<0.05$) while the control groups were not able to decrease the wound area by the same percentage (14.34 ± 7.82% for the untreated control group, 13.06 ± 8.88% for the vehicle control group and 14.98 ± 6.57% for the empty MS control group). By days 7 and 11 post-wounding, rhEGF MS showed a higher decrease ($p<0.05$) in the wound area (61.06 ± 8.69% by day 7 and 90.29 ± 3.60% by day 11) in comparison to the free rhEGF group (51.46 ± 10.29% by day 7 and 78.97 ± 4.44% by day 11), which received two additional doses of 75 µg rhEGF per week. Moreover, at days 7 and 11, the wound closure rate was significantly higher in rhEGF MS group in comparison with the other groups. Typical wound images obtained from each treatment group at day 7 are shown in Figure 5C. From 14 days after wounding until the end of the assay, statistically significant differences were no longer detected between any of the studied groups (Figure 5A).

3.4.3. Re-epithelisation

At day 7 after wounding, in the rhEGF MS group re-epithelisation starts, partially covering the wound surface (according to Sinha et al. 2003 criteria; 1.13 ± 0.35). Nevertheless, the other experimental groups showed smaller re-epithelised areas (less than 1.00 in Sinha et al. 2003 criteria). By day 11, the re-epithelisation was complete but of irregular thickness in the rhEGF MS group (3.25 ± 0.89) while, the untreated control group had an immature complete re-epithelised area (3.88 ± 0.35). However, in

the free rhEGF group (2.38 ± 0.93), the vehicle group (2.88 ± 1.46) and the empty MS group (2.75 ± 1.39), the new epithelia did not cover the entire wound surface. 17 days after wounding, all groups showed complete and mature re-epithelised areas with a normal thickness (Figure 6).

3.4.4. Inflammation

At day 7, a recovery state between diffuse acute inflammation and predominance of chronic inflammation predominated in the group receiving rhEGF MS, in which fibroblast proliferation prevailed (2.38 ± 0.52) (according to Cotran et al. 2000 criteria). In contrast, the other experimental groups did not leave the acute inflammatory state by day 7 (less than 2.00). The differences between rhEGF MS treated group and the other groups reach statistical significance at that point of the study (one-way ANOVA, $p<0.05$). At day 11th after wounding, the rhEGF MS and untreated control groups showed complete recovery of the wound (Score 4). Conversely, other experimental groups had not healed completely. By the end of the study, all the groups showed completed resolution of the injury (Figure 7).

4. Discussion

Non-healing chronic diabetic foot ulcers are characterised by an atypical ECM, re-epithelisation disturbance and a recurrent inflammatory response. Growth factors, such as rhEGF, are essential for correct dermal and epithelial regeneration. However, it has a short half-life *in vivo* and requires multiple administrations, restricting its clinical use (Huang, et al. 2008). To overcome this problem, we propose an alternative protective technique, based on PLGA-Alginate MS for the treatment of chronic wounds, which retains rhEGF biological activity and maintains its sustained release on wound surface.

NaCl. alginate and PLGA, widely used in a broad range of pharmaceutical and biopharmaceutical products (Wenk, et al. 2009), were added in order to optimise the MS properties, primarily with the aim of increasing EE. For the optimisation of the MS, the formulations were prepared using 0.1% rhEGF instead of using a model protein, as previously reported in other studies. (Gutierro, et al. 2002). This delivery system has the advantages of a polymeric therapeutic approach, including biocompatibility, biodegradability and allows a controlled and sustained release of the entrapped drug (Jain 2000; Tessmar and Göpferich 2007).

The current study found that the addition of NaCl and alginate did not produce significant changes in MS size or zeta potential. Although these results differed from Mata. et al. 2011 studies, in which MS loading SPf66 or S3 peptides were prepared (Mata, et al. 2011), they are in agreement with Salvador et al. 2012 results (Salvador, et al. 2012), where no relationship was found between the incorporation of alginate in BSA loading MS and the increase of the negative zeta potential. Interestingly, the encapsulation efficiency was increased after the incorporation of NaCl into the surfactant solution and the solvent removal phase. This increment may be explained by the osmotic pressure increase in surfactant solution and solvent removal phase caused by NaCl, which prevents rhEGF diffusion from the microsphere and becomes MS homogeneous, smooth and regular (Bilati, et al. 2005; Chen, et al. 2004; Perugini, et al. 2001). Moreover, the addition of alginate increased EE to 88.11 ± 1.51% due to an increment of the viscosity in the internal aqueous phase (w_1) which limits the diffusion of rhEGF from the MS and improves the protein–polymer affinity. These data are in agreement with previous work that showed a significant increase in the SPf66 peptide EE after encapsulation in PLGA-Alginate MS (Mata, et al. 2011). The *in vitro* release studies conducted with MS3 and MS4 showed a similar profile with a sustained rhEGF

release for 41 days. Taking into account that the MS properties did not differ between formulations and that the encapsulation efficiency was greater in alginate-containing MS (MS4), the target loading increase to 1%, γ-sterilisation, and further *in vitro*/*in vivo* studies were only carried out in the alginate-containing formulation.

The increment in rhEGF target loading, did not affect particle size, zeta potential and particle morphology. However, the EE decreased when the amount of encapsulating rhEGF raised. This finding may be explained by the increased diffusion of rhEGF to the external water phase of the w/o/w emulsion when the loading was increased from 0.1% to 1%. Furthermore, the increase of the amount of rhEGF released in the first 30 minutes (when the surface associated peptide was released) observed in the *in vitro* release studies of PLGA-Alginate MS and MS4, demonstrate the direct correlation between the increase of rhEGF loading and its distribution on the particle surface. Similar results were also described by Wang et al. 2005, in which the SAP was improved in the formulation with a higher amount of rhEGF encapsulated (Wang, et al. 2005). Regarding to the γ-sterilisation of MS (PLGA-alginate MS-γ), particle size, zeta potential and particle morphology were not affected by the sterilisation process, nevertheless, a statistically significant reduction of EE and a reduced burst effect were detected after γ-sterilisation. These findings are in agreement with Fernández-Carballido et. al 2006, who attributed this decrease to a degradation of the drug during the sterilisation process (Fernández-Carballido, et al. 2006).

With regard to the *in vitro* proliferation and bioactivity assays, the results showed that rhEGF maintains its bioactivity after the microencapsulation and γ-sterilisation processes. This view is supported by He et al. 2011, who demonstrated the stability of recombinant human erythropoietin after its microencapsulation in PLGA MS (He, et al.

2011) and Igartua et al. 2008 who demonstrated that SPf66 integrity was maintained after γ-sterilisation (Igartua, et al. 2008). *In vitro* cell migration studies demonstrated that in presence of rhEGF, either free or obtained from release studies of PLGA-Alginate MS, the wound area was reduced more and faster than in the control group, illustrating that rhEGF maintains its biological activity when it is incorporated into the MS. In contrast to these results, cells used in control group changed cell morphology, losing their fusiform and elongated form due to the absence of rhEGF in the culture media.

The present study shows that the administration of a single dose of rhEGF MS accelerates the resolution of the inflammatory process and wound closure (in terms of contraction and re-epithelisation) of full-thickness wounds in the streptozocin-diabetised Wistar rats compared to multiple doses of non-encapsulated rhEGF or different control groups. Remarkably a single administration of 75 µg of rhEGF MS significantly improved wound closure, re-epithelisation process and inflammatory resolution throughout 11 days in comparison to the 5 doses of 75 µg of non-encapsulated rhEGF (2 per week). Wound restoration was also significantly greater in rhEGF MS than in the untreated control, vehicle control and empty MS control groups. As confirmed by the *in vivo* data, the increased healing effect of rhEGF MS was observed in the first days of the study, at day 4 for the wound closure rate, between 7-11 days for re-epithelisation and at day 7 for inflammatory resolution. These findings confirm the following: (i) microencapsulation into a PLGA-Alginate MS protects the growth factor from the wound environment, making it less vulnerable to protease inactivation and oxidative stress present in the wound area (Chen, et al. 1997; Hardwicke, et al. 2011). Moreover, it is likely that rhEGF released from EGF MS is still active 11 days after wounding. (ii) microencapsulation provides sustained release of

rhEGF, potentially allowing a reduction in the dosage and number of administrations in rhEGF therapy. A simplified dosage regimen would be a major breakthrough for future clinical application of EGF. Therefore, sustained release could increase rhEGF bioactivity levels in the wound area, thereby inducing REGF phosphorylation, which stimulates fibroblast and keratinocyte proliferation and differentiation. (iii) continuous local administration of free rhEGF does not improve wound healing. Some studies attribute this observation to a maximal activity of rhEGF in the range of ng/ml as opposed to µg/ml (Duncan 2003).

However, the improved healing effect induced by rhEGF MS loses its statistical significance over time. This could be attributed to the following two reasons: (i) rhEGF accelerates the healing process only at the initial stage of wound repair, indicating that rhEGF may be mostly required just in the early stages of the healing process (Choi, et al. 2008). This statement is in agreement with Shirakata et al. 2000 which suggest that rhEGF stimulates proliferation of keratinocytes for only 24 hours after its addition (Shirakata, et al. 2000). (ii) streptozocin administration, which ablates pancreatic cells, causes the animal to enter a diabetic state (Takasu, et al. 1991). This model, although useful, does not accurately reflect the compromised healing process in chronic human wounds. Therefore, diabetes, along with aging or obesity, is only one factor that alters and prolongs the healing process. It has been reported that STZ-treated rats do not necessarily suffer a healing delay as seen in chronic wounds (Davidson 1998; LeGrand 1998; Michaels, et al. 2007, Ansell, et al. 2012). This may explain why the differences between the treated and control groups diminished as the study proceeded.

5. Conclusions

In this study, rhEGF-loaded PLGA-Alginate MS were optimised. We have demonstrated the technological advantages of NaCl and alginate incorporation during the PLGA MS preparation showing the suitability of combining two biodegradable and biocompatible polymers such as PLGA and alginate for achieving a greater EE and appropriate release parameters. *In vitro* experiments showed that contact with the encapsulated rhEGF induced the fibroblasts to proliferate and migrate. Moreover, no loss of bioactivity was found after the microencapsulation or γ-sterilisation processes. Finally, the *in vivo* results demonstrate the promising potential use of rhEGF-loaded MS (PLGA-Alginate MS) to promote faster and more effective wound healing in terms of wound contraction, epidermal regeneration and inflammatory stage recovery. It is important to acknowledge that the STZ-treated animals are limited in their ability to imitate human chronic wound healing. Because of this, further experiments are needed to establish an animal model that better fits the human chronic wound healing process.

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Captions

Table 1: Optimisation of rhEGF loaded microspheres. The table describes the composition of the internal aqueous phase, surfactant solution and solvent removal phase of the developed formulations in the MS optimisation process.

Table 2: Protocol for *in vivo* studies in STZ-diabetised Wistar rats. The table describes the treatments administration intervals and the variables measured in all experimental groups: wound area, weight and glycaemia.

Video 1: *In vitro* migration healing assay. The groups studied were: fresh serum free medium, 15 ng/mL rhEGF obtained from the release tests (PLGA-Alginate MS) on serum free supplemented DMEM medium and 15 ng/ml free rhEGF on serum free DMEM medium. The video shows that rhEGF treated groups closed scratch assay wounds more rapidly than the control group. Original magnification × 40.

Figure 1: Physico-chemical characterisation of the MS during the optimisation process. (A) MS size, zeta potential and EE. Data shown as mean ± S.D. (B) SEM photographs of MS1, MS2, MS3, MS4 formulations. Scale bars in (MS1-4) denote 10µm (original magnification x2500). (C) rhEGF cumulative *in vitro* release profiles from MS3 and MS4 microspheres. The results are given in terms of the cumulative percentage of rhEGF released over time.

Figure 2: Physico-chemical characterisation of the PLGA-Alginate MS and PLGA-Alginate MS-γ. (A) MS size, zeta potential and EE. Data shown as mean ± S.D. Statistical significance is expressed as *$p<0.05$. (B) SEM photographs of PLGA-Alginate MS and PLGA-Alginate MS-γ. Scale bars denote 10µm (original magnification x1000). (C) rhEGF cumulative *in vitro* release profiles from PLGA-Alginate MS and PLGA-Alginate MS-γ. The results are given in terms of the cumulative percentage of rhEGF released over time. PLGA-Alginate MS-γ showed a reduced burst effect resulting in a statistically different release profile.

Figure 3: Bioactivity assay for rhEGF. The results are given as 50% effective dose (ED_{50}) of the free rhEGF, rhEGF released from PLGA-Alginate MS and rhEGF released from PLGA-Alginate MS-γ measured in Balb/C 3T3 cells. Data shown as mean ± S.D. One way ANOVA, ($p<0.05$)

Figure 4: *In vitro* migration assay. Images obtained at 0, 5, 18 and 24 hours after wound creation rhEGF treated groups closed scratch assay wounds more rapidly than the control group. (A) fresh serum free medium, (B) 15 ng/mL rhEGF obtained from the release tests (PLGA-Alginate MS) on serum free supplemented DMEM medium and (C) 15 ng/ml free rhEGF on serum free DMEM medium. Original magnification × 40.

Figure 5: *In vivo* study of wound closure. (A) Wound closure calculated as a percentage area of the original wound during the experiment. Data shown as mean ± S.D. (B) wound closure during the experiment. rhEGF MS treated groups heal before than the others experimental groups. (C) wound images obtained from each treatment group at day 7 of the experiment.

Figure 6: *In vivo* study of re-epithelisation. Evaluation of re-epithelisation grade was made on day 7, 11 and 17. (A) untreated control, (B) vehicle control, (C) empty MS control, (D) free rhEGF group and (E) rhEGF MS group. By day 7 re-epithelisation took place in rhEGF MS group. By day 11 in rhEGF MS and untreated control groups there was an immature re-epithelised area covering the entire wound surface. For the rest of the experimental groups the re-epithelisation was not complete until the end of the study.

Figure 7: *In vivo* study of restoration of the inflammatory process. Inflammatory recovery was evaluated on day 7, 11 and 17 after wounding. Data shown as mean ± S.D. Statistical significance is expressed as *$p<0.05$ compared to untreated control, vehicle control, empty MS control and free rhEGF groups. rhEGF-MS treated groups left the acute-inflammatory phase at the 7th day. By the 11th day after wounding rhEGF-MS and untreated control showed a complete recovery and healing. In contrast the other experimental groups did not show a complete recovery of the inflammatory state until the end of the study.

Table 1.

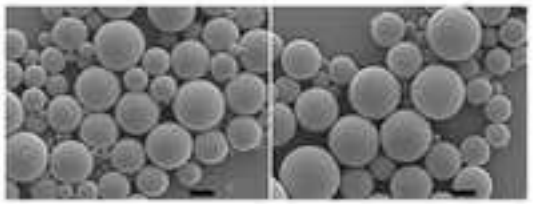
Formulation		Optimization of rhEGF loaded microspheres		
Name	Target drug loading (% w/w)	Internal aqueous phase	Surfactant solution	Solvent removal phase
MS1	0.1	100 µg rhEGF, 5 mg HSA and 0.5 mg PEG 400 in 0.2 ml	15 ml 5% (v/v) PVA aqueous solution	400 ml milliQ water
MS2	0.1	100 µg rhEGF, 5 mg HSA and 0.5 mg PEG 400 in 0.2 ml	15 ml 5% (v/v) PVA and 5% NaCl aqueous solution	400 ml milliQ water
MS3	0.1	100 µg rhEGF, 5 mg HSA and 0.5 mg PEG 400 in 0.2 ml	15 ml 5% (v/v) PVA and 5% NaCl aqueous solution	400 ml 5% (w/v) NaCl aqueous solution
MS4	0.1	100 µg rhEGF, 5 mg HAS, 0.5 mg PEG 400 in 0.1 ml and 0.1ml of 2% (w/w) sodium alginate MVG aqueous solution	15 ml 5% (v/v) PVA and 5% NaCl aqueous solution	400 ml 5% (w/v) NaCl and 0.6 mM $CaCl_2$ aqueous solution
PLGA-Alginate MS rhEGF-MS	1	1 mg rhEGF, 5 mg HAS, 0.5 mg PEG 400 in 0.1 ml and 0.1ml of 2% (w/w) sodium alginate MVG aqueous solution	15 ml 5% (v/v) PVA and 5% NaCl aqueous solution	400 ml 5% (w/v) NaCl and 0.6 mM $CaCl_2$ aqueous solution

Table 2.

Experimental groups	Time (days)					
	0	4	7	11	14	17
Untreated control Vehicle control Empty MS Control Free rhEGF rhEGF MS (PLGA-Alginate MS) Drug loading: 1% (w/w)	**DAY 0** 1. Animals wounded 2. Weighed and glycaemia measurement 3. Digital photography and wound area drawing 4. Treatments application					
		DAYS 4, 7, 11 and 14 1. Digital photography and wound area drawing 2. 75 µg of free rhEGF application				
			DAYS 5, 9, 14 and 17 1. Weighed and glycaemia measurement			
				DAY 17 1. Digital photography and wound area drawing 2. Animal euthanasia		

MS PREPARATION AND CHARACTERIZATION

rhEGF PLGA-Alginate MS



***IN VITRO* CELL CULTURE STUDIES**

Effect of rhEGF on cell proliferation

Bioactivity assay of rhEGF

***In vitro* wound healing assay**

***IN VIVO* STUDIES**