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Chitosan-based dressings loaded with neurotensin—an efficient strategy to improve early diabetic wound healing

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ABSTRACT

One important complication of diabetes mellitus is chronic, non-healing diabetic foot ulcers (DFUs). This study aims to develop and use dressings based on chitosan derivatives for the sustained delivery of neurotensin (NT), a neuropeptide that acts as an inflammatory modulator in wound healing. Three different derivatives, namely N-carboxymethyl chitosan, 5-methyl pyrrolidinone chitosan (MPC) and N-succinyl chitosan, are presented as potential biomaterials for wound healing applications. Our results show that MPC has the best fluid handling capacity and delivery profile, also being non-toxic to Raw 264.7 and HaCaT cells. NT-loaded and non-loaded MPC dressings were applied to control/diabetic wounds to evaluate their in vitro/in vivo performance. The results show that the former induced more rapid healing (50% wound area reduction) in the early phases of wound healing in diabetic mice. A NT-loaded MPC foam also reduced expression of the inflammatory cytokine TNF-α ($P < 0.001$) and decreased the amount of inflammatory infiltrate on day 3. On day 10 MMP-9 was reduced in diabetic skin ($P < 0.001$), significantly increasing fibroblast migration and collagen (COL1A1, COL1A2 and COL3A1) expression and deposition. These results suggest that MPC-based dressings may work as an effective support for sustained NT release to reduce DFUs.

1. Introduction

Diabetes mellitus is one of the most prevalent chronic diseases world wide. Impaired wound healing is a complication of diabetes that results in a failure diabetic foot ulcers (DFUs) to completely heal [1]. Complications of DFUs lead to frequent hospitalization and, in extreme cases, to amputation, resulting in high hospital costs and a poor quality of life for patients [2]. DFUs are a multifactorial complication that result particularly as a consequence of peripheral neuropathy, impaired vascular function, impaired angiogenesis and/or chronic inflammation [1,3].

Recently it became evident that the peripheral nerves and cutaneous neurobiology contribute to wound healing [4]. Loss of peripheral sensory and autonomic nerves reduces the production of neuropeptides that are important for proper wound healing [3]. Neurotensin (NT) is a bioactive neuropeptide that is widely distributed in the brain and in several peripheral tissues [5,6]. NT interacts with leukocytes, mast cells, dendritic cells and macrophages, leading to cytokine release and chemotaxis, which can modulate the immune response. In addition, NT affects microvascular tone, vessel permeability, vasodilation/vasoconstriction and new vessel formation, helping to improve angiogenesis during wound healing [3,7,8].

Some studies have demonstrated that topical application of neuropeptides, such as substance P and neuropeptide Y, can improve wound healing in diabetes [9,10]. However, the major problem with topical administration of peptides is their short half-life and loss of bioactivity in the peptidase-rich wound environment [11]. An alternative strategy to overcome this problem is the use of biocompatible wound dressings for the sustained delivery of neuropeptides. These dressings should, however, also replicate the characteristics of skin in order to promote the proliferation and migration of fibroblasts and keratinocytes, as well as to enhance collagen synthesis, leading to proper healing with little scar formation [12,13].

Wound dressings based on natural polymers have been extensively applied to simulate extracellular matrix (ECM) regeneration

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after injury [12,13]. One of the most used naturally based polymers for wound healing applications is chitosan [12], which is a linear co-polymer of D-glucosamine and N-acetyl-D-glucosamine [14]. Since it is derived from chitin, a polymer found in fungal cell walls and the crustacean exoskeleton, it is a relatively inexpensive and abundant material [15]. In addition, it has proven to be biodegradable, biocompatible, non-antigenic, non-toxic, bioadhesive, antimicrobial, bioactive and to have hemostatic capacity [15–17]. Furthermore, chitosan promotes tissue granulation and accelerates wound healing through the recruitment of inflammatory cells such as polymorphonuclear leukocytes (PMN) and macrophages to the wound site [18].

To increase its poor solubility in water chitosan functional groups can be chemically modified to produce water-soluble chitosan derivatives such as N-carboxymethyl chitosan (CMC), 5-methyl pyrrolidinone chitosan (MPC) and N-succinyl chitosan (SC) [19–21]. These chitosan derivatives are functional biomaterials that maintain the antibacterial and non-cytotoxic properties of the parent chitosan. In addition, they stimulate the extracellular lysozyme activity of skin fibroblasts [22,23].

The aim of this study was to develop and apply wound dressings prepared from the chitosan derivatives referred above (CMC, MPC, SC) for the prolonged and efficient delivery of NT to diabetic and non-diabetic wounds, while also conferring wound protection and comfort. The progression of skin wound healing in diabetic and non-diabetic mice was also evaluated by analysis of the inflammatory and angiogenic effects of NT when applied to skin wounds alone or loaded into MPC-based dressings.

2. Materials and methods

2.1. Materials

Chitosan (medium molecular weight, 90% degree of acetylation confirmed by ^{1}H NMR), glyoxylic acid monohydrate (98%), sodium hydroxide, sodium borohydride (99.5%), levulinic acid (98%), succinic anhydride (97%), reduced glutathione (GSH), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), dialysis membranes (Spectra/Por 6) with a MWCO of 8000 Da and methanol p.a. were obtained from Sigma–Aldrich (USA). Acetic acid was obtained from Panreac (Spain), and ethanol was purchased from Riedel-de-Haen (Germany). Ketamine (Clorketam 1000) was obtained from Vétoquinol (Portugal) and xylazine (Rompun) from Bayer HealthCare (Germany). NT was purchased from Bachem (Switzerland). The antibodies against tumor necrosis factor-α (TNF-α) and metalloproteinase 9 (MMP-9) were purchased from Cell Signaling Technology (USA) and the antibodies against vascular endothelial growth factor (VEGF) and actin were purchased from the Millipore Corp. (USA).

2.2. Synthesis of the chitosan derivatives CMC, MPC and SC

Chitosan (2 g) was reacted with glyoxylic acid (1.16 g), levulinic acid (5 ml) or succinic anhydride (3 g) to synthesize CMC, MPC and SC, respectively [24,25], following by precipitation with ethanol and dialysis to remove unreacted reagents. Foams of CMC, MPC and SC were prepared by freeze-drying, adding 1.5 ml of each solution to 12-well plates. The average thickness of the materials obtained was 250 ± 15 μm. All samples were stored at −20 °C, away from light and humidity, before use. The degree of substitution of each of the derivatives was calculated by ^{1}H NMR using a Bruker Avance III 400 MHz spectrometer with a 5 mm TIX triple resonance detection probe using D_2O acidified with acetic acid (10 μl of acetic acid in 600 μl of D_2O).

2.3. Scanning electron microscopy (SEM)

SEM micrographs were obtained at 5 keV (JEOL model JSM-5310, Japan). Samples were coated with gold (approximately 300 Å) in an argon atmosphere.

2.4. Water vapor and water sorption capacities

Samples of CMC, MPC and SC, 22 mm in diameter, were dried at 37 °C for 72 h until a constant weight was achieved. Both water vapor and water sorption capacities were measured gravimetrically. In the first case dried foams were exposed to a 95% relative humidity atmosphere, in a desiccator containing a saturated solution of potassium sulfate at 32 °C according to Dias et al. [26]. In the second case samples were immersed into phosphate buffer (pH 7) at 37 °C and weighed after removing the surface phosphate buffer using filter paper.

Samples were weighed at fixed time intervals until they reached equilibrium. The water vapor and water sorption capacities were calculated as the ratio between the sample weight at time t and the initial sample dry weight. All samples were measured in duplicate.

2.5. In vitro release kinetics

Kinetic release profiles of GSH were determined spectrophotometically (Jasco model 630, Japan) at 412 nm. Known amounts of GSH solution (5 mM) were loaded into previously weighed samples of each polymer. The GSH solution had been previously placed in an ultrasonic bath to prevent oxidation. After drying the samples were immersed in phosphate buffer at pH 6, 7 or 8 at 32 °C with orbital stirring (100 r.p.m.) for 8 h. The quantification of GSH release was based on the Ellman's test. This test is based on the addition of DTNB, a yellow water-soluble compound that reacts with free sulfhydryl groups in peptide solutions. At predetermined time points an aliquot (100 μl) of the released solution was removed and analyzed using a mixture of 1800 μl of phosphate buffer and 100 μl of DNTB stock solution (20 mM). 100 μl of fresh phosphate buffer was added to the medium each time point. Each sample was analyzed in duplicate.

2.6. Cell culture

Mouse leukemic monocyte macrophages (Raw 264.7) and human keratinocyte (HaCaT) cells were cultured in DMEM (Dulbecco's modified Eagle's medium), pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 3.02 g l^{-1} sodium bicarbonate, 30 mM glucose, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin at 37 °C in a humidified incubator containing 5% CO_2. Sub-culturing was performed according to ATCC recommendations. The Raw 264.7 and HaCaT cell lines were purchased from the ATCC (No. TIB-71) and CLS (No. 300493), respectively.

2.7. MTT assay

Raw 264.7 (8×10^4 cells per well) and HaCaT (4×10^4 cells per well) cells were plated individually in 12-well plates with 430 μl of DMEM above the previously sterilized (UV light for at least 30 min) biomaterials. After 24 and 48 h incubation 43 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg ml^{-1}) was added to each well. The plates were further incubated at 37 °C for 1 h in a humidified incubator containing 5% CO_2. After this period 300 μl of acidic isopropanol (0.04 N HCl in isopropanol) was added. Quantification was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a