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Effect of different chitosan derivatives on *in vitro* scratch wound assay: A comparative study

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ABSTRACT

Different strategies have been developed to make the wound-healing process faster and less painful. Recently, numerous studies demonstrated the ability of chitosan to accelerate wound healing. Aim of the present study has been to evaluate the effect of different chitosan derivatives to improve wound healing process. Quaternary ammonium–chitosan conjugates with low or high molecular weight (MW) and their thiolated derivatives effect were studied on human skin fibroblasts in terms of viability and migration (scratch wound assay). Results were compared both with basal medium (untreated cells) and with a positive control (chitosan chlorhydrate). After 24 h both high and low MW chitosan derivatives were non-toxic up to 10 μg/ml. The concentration of 10 μg/ml was used for wound healing experiments. High-MW quaternary ammonium–chitosan conjugates bearing thiol groups on their chains were more effective in promoting cell migration than the non-thiolated conjugates and the chitosan chlorhydrate. Moreover, they significantly improve wound healing process compared to untreated cells.

According to the present *in vitro* preliminary results, high MW thiolated quaternary ammonium–chitosan conjugates can be considered good candidates for the management of wounds.

1. Introduction

According to the Wound Healing Society, a wound is the result of "disruption of normal anatomic structure and function" [1] and may be classified as acute or chronic, on the basis of the wound healing process [2,3]. During this process, growth factors released from fibroblasts, macrophages, neutrophils, keratinocytes, and endothelial cells influence all phases of wound healing and act by providing signals for various cellular activities [2,4,5]. Fibroblasts, one of the dominant components of dermal structure, play an important function all through the process. In fact, in the early stage of wound healing, they migrate to the traumatized region to promote the regeneration of blood vessels, extracellular matrix deposition and granulation tissue formation, through the release of some angiogenesis factors [6]. In the advanced trauma repair, a large number of fibroblasts mature into myofibroblasts, which promote wound closure [7,8]. Different strategies have been developed to make the wound healing process faster and less painful. Recently, numerous studies demonstrated the function of chitosan as a wound healing accelerator [9–11].

Chitosan, a polysaccharide obtained by partial deacetylation of natural chitin, has been widely used as a wound dressing material due to its properties [12]. The notable properties of chitosan include its non-toxicity, hemostatic action, anti-inflammatory effect, biodegradability, biocompatibility, antimicrobial activity, retention of fibroblast growth factors, and release of glucosamine [12,13]. Chitosan and its derivatives could accelerate wound healing by enhancing the functions of inflammatory cells, such as fibroblasts, polymorphonuclear leukocytes and macrophages [14]. The wound healing effects of chitosan could be affected by its physico-chemical characteristics, such as molecular weight (MW), deacetylation degree and derivatization [12,15].

The application of native chitosan is limited by non-solubility in neutral or alkaline media. Therefore, new forms of chemically modified chitosan have been developed in order to improve the beneficial properties of this biomaterial. In particular, chitosan derivatives soluble in aqueous neutral or alkaline media have

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been developed, such as chitosan derivatives containing quaternary ammonium salts [16,17] and carboxymethyl chitosan [18].

The purpose of this study is to compare the effects of 8 chitosan derivatives with different MWs, either thiolated or not, on the wound healing process. For the purpose of sparing animals the *in vitro* scratch assay was used to assess the wound healing rate, following a procedure described in the literature [19].

2. Materials and methods

2.1. Preparation of chitosan derivatives

The commercial chitosan (Ch) had an average viscometric MW of 590 kDa and a deacetylation degree, determined by IR or NMR, of 90% or 82% [20]. Its MW was reduced by oxidative depolymerization (see, *e.g.*, Refs. [21,22]), to obtain rCh (viscometric MW, 32 kDa). The viscometric MWs of Ch and rCh were determined by an Ostwald U-tube capillary viscometer (Cannon-Fenske series ASTM 75), following the procedure reported by Khalid et al. [23]. Quaternary ammonium–Ch or quaternary ammmmonium–rCh conjugates (N^+-Ch or N^+-rCh) were synthesized by reacting diethylaminoethyl chloride hydrochloride with Ch or rCh, through the materials and procedure described by Zambito et al. [24,25], keeping the pH at 8 and controlling the temperature at 50 °C (product code, N^+-Ch(50°) or N^+-rCh(50°)), or 60 °C (product code, N^+-Ch(60°) or N^+-rCh(60°)). Thiolation of N^+-Ch and N^+-rCh was carried out by attaching thioglycolic acid to unsubstituted primary amino groups still present on the polymer chains, *via* formation of amide bonds mediated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [25]. The degree of acetylation (DA) of chitosans, the degree of substitution (DS) of the quaternary ammonium–chitosan conjugates by pendant moieties containing quaternary ammonium groups, and the number of quaternary ammonium groups in substituted moieties (n), were determined by NMR [20,24]. The degree of substitution by thiol-bearing groups (ThDS) was determined by iodometry [26]. The above characteristics of chitosans and chitosan derivatives are summarized in Table 1.

2.2. Cell culture and treatment

Fibroblasts were isolated from human skin. Cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Medium was routinely changed every 3rd day and at confluence cells were subcultured (split ratio 1:3) by trypsinization (0.5% trypsin/0.02% EDTA). In all experiments cells were used between P4 and P5 passage cultures.

Table 1
Chemical characteristics of chitosans and chitosan derivatives.

Polymer	DA (%)	DS (%)	n	ThDS (%)
Ch	17.5	–	–	–
rCh	11.3	–	–	–
N^+-Ch(50°)	–	21.1	4.1	–
N^+-Ch(60°)	–	59.2	1.7	–
N^+-rCh(50°)	–	26.4	3.8	–
N^+-rCh(60°)	–	55.3	2.1	–
N^+-Ch(50°)-SH	–	–	–	4.3
N^+-Ch(60°)-SH	–	–	–	0.5
N^+-rCh(50°)-SH	–	–	–	2.8
N^+-rCh(60°)-SH	–	–	–	1.4

DA, degree of acetylation; DS, degree of substitution; n, number of quaternary ammonium groups in substituted moieties; ThDS, degree of substitution by thiol-bearing groups.

2.3. Cytotoxic activity

The cytotoxicity of chitosan derivatives was examined by WST-1 assay, based on the cleavage of tetrazolium salt (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate, Roche Applied Science, Mannheim, Germany) by mitochondrial dehydrogenases, present in viable cells. A stock solution of each chitosan derivative (1 mg/ml) was prepared by dissolving 5 mg of polymer in 5 ml of sterile water. Each stock solution was then diluted to 5, 10, 30, 50, or 100 μg/ml with DMEM plus 1% FBS, the pH of which was first adjusted to 4.0, by 5 M HCl, then to 7.0 by 6 M NaOH. Cells were seeded on a 96-well cell culture plate at a cell density of 2×10^3 cells/well (100 μl medium/well). After 24 h of cultivation at 37 °C, 100 μl of each chitosan derivative solution prepared as described above was added to each well and incubated for further 24 h. At the end of the treatment cells were washed with PBS and 100 μl of culture medium containing 10% (v/v) WST-1 reagent (10 μl/well) was added to each well and incubated for 4 h at 37 °C, 5% CO_2. Absorbance of the medium at 450 nm was measured using a microplate spectrophotometer (Thermo Scientific). The absorbance directly correlated to the number of metabolically active cells. Absorbance was plotted as percent absorbance of control (culture medium without chitosan derivatives).

2.4. In vitro *scratch wound healing assay*

Fibroblasts between passages 4 and 5 were seeded in 24-well plates (12×10^3 cells/well) and grown until confluence in complete DMEM with 20% FBS in a humidified atmosphere of 5% CO_2. Thereafter, the procedure described by [19] was followed. A straight scratch was made with a P200 pipette tip, to simulate a wound. The cell debris was removed and the edge of the scratch was smoothed by washing with serum free medium. The wound was exposed to high and low MW chitosan derivatives at the concentration of 10 μg/ml in DMEM with 1% serum for 24 h at 37 °C. The cells without treatments were used as the control. The closure of the scratch was observed under a microscope (5× magnification). At 0 and 24 h after wounding, digital images of cells were captured by a phase contrast microscope (Nikon) equipped with a digital CCD camera (EOS 1000D, Canon, Milano, Italy). To quantify the closure of the scratch the difference between wound width at time 0 and time 24 h was determined. Each well was marked below the plate surface by drawing a vertical line, to allow identification of the same scratched area in order to take consistent pictures. Scratch area was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Migration rate was expressed as percentage of scratch closure on an initial area basis, according to the following equation:

$$\text{Scratch closure rate} = \left[\frac{(At_0 - At)}{At_0}\right] \times 100$$

where At_0 is the scratch area at time 0, and At is the correspondent scratch area at 24 h. The values shown are the means of three wells from three independent experiments.

2.5. Statistical analysis

Data are presented as means ± SD of at least three independent experiments. Comparisons are made by the Student's t-test or by ANOVA when appropriate. Differences are considered statistically significant at $P < 0.05$. Statistical analysis was carried out using StatView™ 5.0 software (SAS Institute, Cary, NC, USA).