Effect of chitosan derivatives on *in vitro* wound-healing process of human skin fibroblasts

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Abstract

Background: 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 new chitosan derivatives to improve wound healing process.

Methods: Quaternary ammonium-chitosan conjugates with low or high MW and their thiolated derivatives were used. Human skin fibroblasts were isolated and cell viability was assessed by incubating fibroblasts with different concentrations of chitosan derivatives (5, 10, 50, or100 μg/ml) for 24 h. The wound healing experiment was performed by scratching a confluent cell monolayer, thus simulating a wound. Chitosan derivatives were added to the cells and wound healing process was monitored under a microscope at 0 and 24 h.

Results: After 24 h both high and low MW chitosan derivatives were non-toxic up to 10 μg/ml. In particular, the thiolated and non-thiolated low MW derivatives were non-toxic up to 50 and 100 μg/ml, respectively. The concentration of 10 μg/ml was used for wound healing experiments. Wound healing was accelerated by thiolated high MW chitosan derivatives

Conclusion: According to the present *in vitro* preliminary results, high MW thiolated quaternary ammonium-chitosan conjugates can significantly improve wound healing process, hence, they can be considered good candidates for management of wounds.

Keywords: Chitosan; fibroblast; molecular weight; wound healing

Abbreviations

Ch; chitosan

CMC; carboxymethyl chitosan

DEAE; diethylaminoethyl

DMEM; Dulbecco's modified eagle medium

EDAC; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

FBS; fetal bovine serum

MW; molecular weight

TGA; thioglycolic acid

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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 are 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, 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 acqueous neutral or alkaline media have been developed, such as chitosan derivatives containing quaternary ammonium salts [16,17] and carboxymethyl chitosan [18].

Purpose of this study is to put to comparison 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 [REF].

Materials and methods

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% [19]. Its MW was reduced by oxidative depolimerization (see, e.g., refs. 20, 21), 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. [M.N. Khalid, L. Ho, F. Agnely, J.L. Grossiord, G. Couarraze Swelling properties and mechanical characterization of a semi-interpenetrating chitosan/polyethylene oxide network. Comparison with a chitosan reference gel STP Pharma Sciences, 9 (1999), pp. 359–364] 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. [22, Zambito, Y., Zaino, C., Uccello-Barretta, G., Balzano, F., Di Colo, G. (2008). Improved synthesis of quaternary ammonium-chitosan conjugates (N^+-Ch) for enhanced intestinal drug permeation. *European Journal of Pharmaceutical Sciences*,33, 343-350.], 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 [22]. The degree of acetylation (DA)of chitosans, the degree of substitution of the quaternary ammonium-

chitosan conjugates by pendant moieties containing quaternary ammonium groups(DS), and the number of quaternary ammonium groups in substituted moieties (n), were determined by NMR [19, Zambito, Y., Zaino, C., Uccello-Barretta, G., Balzano, F., Di Colo, G. (2008). Improved synthesis of quaternary ammonium-chitosan conjugates (N^+-Ch) for enhanced intestinal drug permeation. *European Journal of Pharmaceutical Sciences*,33, 343-350.] The degree of substitution by thiol-bearing groups (ThDS) was determined by iodometry [Kast, C.E., & Bernkop-Schnürch, A. (2001). Thiolated polymers—thiomers: development and in vitro evaluation of chitosan-thioglycolic acid conjugates. *Biomaterials*, 22, 2345-2352.]. The above characteristics of chitosans and chitosan derivatives are summarized in Table 1.

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

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-P5 passage culture.

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 (1mg/ml) was prepared by dissolving 5 mg of polymer in 5 ml of sterile water. Each stock solutions 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. Absorbances were plotted as percent absorbance of control (culture medium without chitosan derivatives).

In vitro scratch wound healing assay

Fibroblasts between passage 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 Tizio et al. [REF] 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 was used as the control. The closure of the scratch was observed under a microscope (5X 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:

$$\text{scratch closure rate} = [(At0-At)/At0]\cdot 100$$

where At0 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.

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 Intitute, Cary, NC, USA).

Results

Chemical characteristics of chitosan derivatives

Data shown in Tab. 1 demonstrate that controlling the reaction temperature at 50° or 60°C resulted in quaternary ammonium-chitosan derivatives very different from one another. Indeed, at 50°C

derivatives with lower quaternization degrees and higher number of quaternary ammonium groups in substituted moieties were obtained, compared with polymers prepared at 60°C. As can be deduced from relevant data in the table, significant fractions of unsubstituted glucosamine units were still present on the polymer chains. These were potentially available for covalent attachment of thiol groups via formation of amide bonds between the primary amino group of glucosamine and the carboxyl group of thioglycolic acid. The thiol content of N^+-Ch(50°)-SH or N^+-rCh(50°)-SH appears in Tab. 1 to be significantly higher of the respective values for N^+-Ch(60°)-SH or N^+-rCh(60°)-SH. This is probably due to the higher number of glucosamine units available for thiolation in the respective parent N^+-Ch and N^+-rCh polymers.

Citotoxicity of low and high MW chitosan derivatives

To determine the highest non-toxic concentrations of Ch and rCh derivatives fibroblasts were incubated for 24 h with 5, 10, 30, 50, 100 μg/ml of each chitosan derivative. Dose-dependent cell viability indicated that in no case were significant cytotoxic effects observed at concentrations below 10 μg/ml (**figure 1 A and B**). Actually, N^+-*r*Ch(50°) either thiolated or not, improved cell viability significantly compared with untreated cells (control) (**figure 1B**). In particular, N^+-*r*Ch(50°) and N^+-*r*Ch(50°)-SH were non-toxic up to 50 and 100 μg/ml, respectively. For their part, both N^+-Ch(50°) and N^+-Ch(50°)-SH were found non-toxic up to 10 μg/ml.

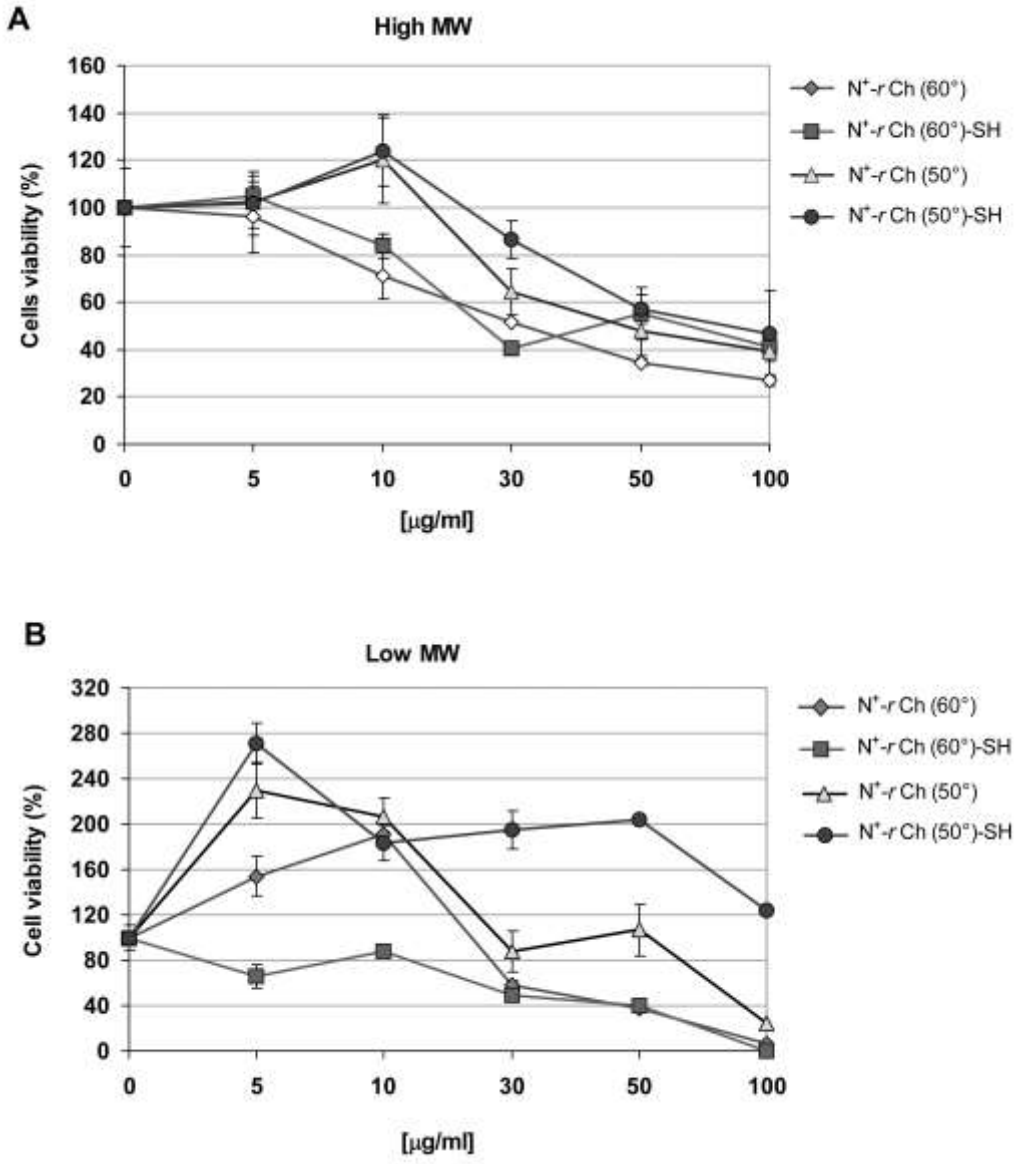

Figure 1. Cell viability. Human fibroblasts viability were assessed by WST-1 assay following ….

Nella legend della Fig. 1A sostituire, nelle sigle, Ch al posto di rCh
Nella Fig. 1A per N^+-Ch(60°) i simboli dei dati nel diagramma non corrispondono a quello nella legend

In vitro scratch wound healing assay

In vitro scratch wound healing assay was carried out to observe the effects of Ch and rCh derivatives on the healing process. It can be observed in **Figure 2** that, in the course of 24-h exposure to either N^+-Ch(50°)-SH or N^+-Ch(60°)-SH, fibroblasts moved toward the opening to close the scratch wound by about 70% and significantly accelerated the wound healing process compared to the control. Finally, as can be observed in **Figure 3,** of the rCh polymers only N^+-

*r*Ch(50°)-SH showed a positive trend to wound healing. On the other hand, no significant effects were observed after 24h of treatment with non-thiolated quaternary ammonium-chitosan conjugates or Ch·HCl or rCh·HCl, used as positive control.

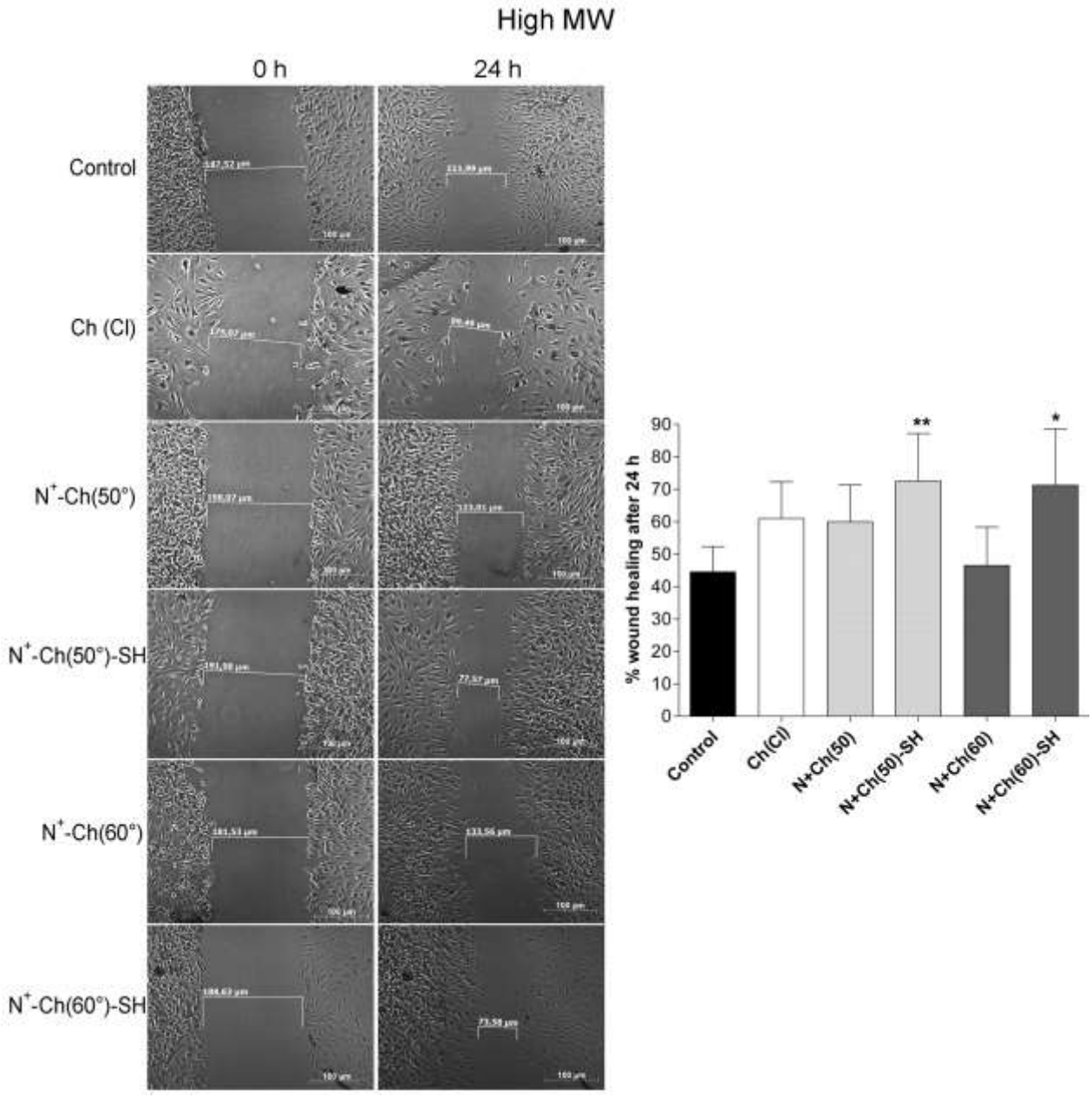

Figure 2. Scratch wound assay. Representative phase contrast micrographs of cells treated with 10 µg/ml Ch derivatives for 0 and 24 h (5X magnification). Bar graphs represent the quantification of the effects of Ch derivatives on scratch wound healing. Bars represent the means ± SD of values obtained from three independent experiments. Wound closure rates are expressed as percentage of scratch closure after 24 h compared to initial area. *P<0.05 and **P<0.01 vs control (untreated cells).

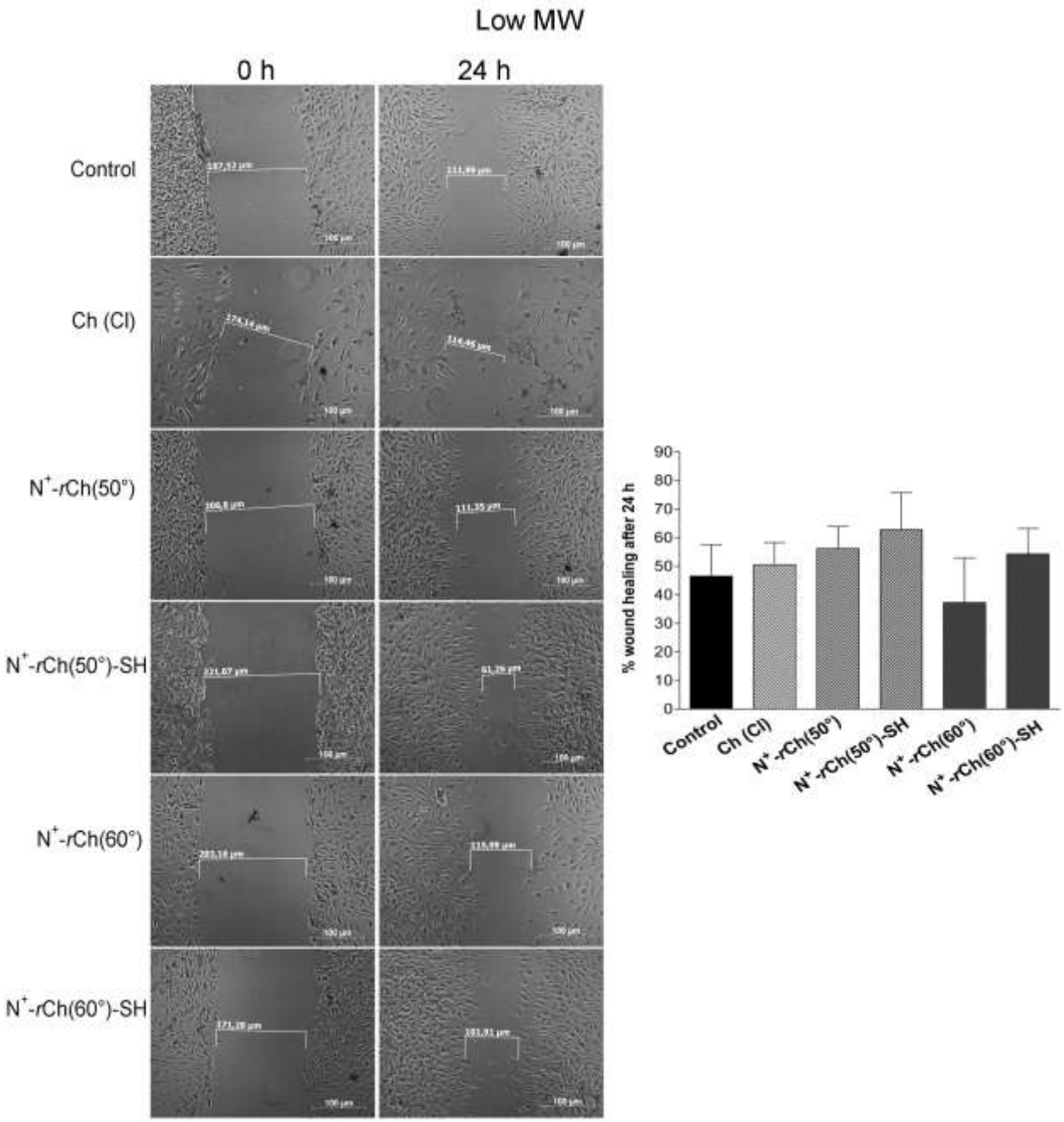

Figure 3. Scratch wound assay. Representative phase contrast micrographs of cells treated with 10 µg/ml rCh derivatives- for 0 and 24 h (5X magnification). Bar graphs represent the quantification of the effects of rCh derivatives on scratch wound healing. Bars represent the means ± SD of values obtained from three independent experiments. Wound closure rates are expressed as percentage of scratch closure after 24 h compared to initial area. Control consists in untreated cells.

Sigla del chitosano commerciale cloridrato: Ch·HCl

Sigla del chitosano depolimerizzato cloridrato: rCh·HCl

Discussion

Wound healing is a complex and dynamic process including inflammation, proliferation and remodeling, in which migration of fibroblasts plays an essential role in wound repair [23]. Chitosan, a derivative of chitin, which is one of the most abundantly found natural polymers, has numerous biomedical applications, in particular, chitosan has been used to promote wound healing [24-26]. In fact, chitosan is suitable for use in wound dressings, as it not only aids the healing process but also, it is biodegradable, biocompatible, non-toxic, bioadhesive, bioactive, non-antigenic, anti-microbial and at the same time it exerts haemostatic effects [9, 27]. However, the application of chitosan is often limited by its poor solubility in physiological media. To improve its water solubility it has been subjected to chemical modifications, such as quaternization, carboxymethylation, etc. [28,29]

It has been demonstrated that chitosan induces fibroblast activation, cytokine production, giant cell migration and stimulation of type IV collagen synthesis by fibroblasts [30]. Shi et al., demonstrated that chitosans promote adequate granulation tissue formation, accompanied by angiogenesis and regular deposition of thin collagen fibers [31]. Natural polymers such as chitosan, collagen and gelatin can successfully be used to fabricate wound dressings with desirable properties, since chitosan and similar substrates are the principal structural components of natural extra cellular matrix (ECM) [33]. Zhang et al. , studied the effects of chitosan with different MWs and carboxymethyl chitosan on the structure and function of clotting-related proteins[25]. They observed that chitosan and fibrinogen can form a complex mainly by electrostatic attraction. In fact, the structure and conformation of fibrinogen are altered by chitosan and carboxymethyl chitosan [25].

The beneficial effects of chitosan and its derivatives depend on physico-chemical properties, such as polymer MW and pH of solution, and chemical ones, such as type of moieties substituted on polymer chains. Wound healing properties of chitosans with different MWs and degrees of deacetylation were examined by Alsarra [I.A. Alsarra, 2009. Chitosan topical gel formulation in the

management of burn wounds, Int. J. Biol. Macromol. 45:16-21.] who found that the strongest wound healing effect corresponded with high MW and deacetylation degree. To note that Seyfarth et al. [32] observed a direct influence of MW on antifungal activity of chitosans, i. e., a low antifungal activity was associated with a low MW. Also the deacetylation degree must have a role in the bioactivity of chitosan. Indeed, Howling et al., who examined the effects of chitin and chitosan with different deacetylation degrees and similar MWs on the proliferation of human skin fibroblasts, reported that chitosans with relatively high deacetylation degree (89 %) strongly stimulated fibroblast proliferation, while samples with lower deacetylation degree (37 %) showed a lesser activity [10]. It was on the basis of the above literature information that we used highly deacetylated chitosans for derivatization, namely, high-MW Ch, deacetylated by 82.5 %; and low-MW rCh, deacetylated by 88.7 %. Of the two, the former promoted the higher mean scratch closure rate, as appears from a comparison of relevant mean values in Figures 2 and 3. Although this difference has a poor statistical significance it is in agreement with the reported influence of MW on chitosan bioactivity [Alsarra, 32]. Derivatization of chitosans into quaternary ammonium-chitosan conjugates, although ineffective for wound healing, was necessary to grant the successively thiolated polymers a pH independent solubility, in turn necessary for the significant wound healing activity appearing in Figure 2 for N^+-Ch(50°)-SH and N^+-Ch(60°)-SH. Reportedly thiol groups are responsible for the enhanced bioactivity of thiolated polymers (thiomers). In fact, thiomers in combination with reduced glutathione were shown to improve the uptake of hydrophilic macromolecules from the GI tract [Bernkop-Schnürch A^1, Kast CE, Guggi D., 2003. Permeation enhancing polymers in oral delivery of hydrophilic macromolecules: thiomer/GSH systems. J. Control. Release, 93:95-103.] Successively, Zambito et al. reported that a chitosan derivative carrying thiol along with quaternary ammonium groups showed an improved ability to enhance drug permeability across the rat intestinal mucosa compared with the non-thiolated polymer [Y. Zambito, S. Fogli, C. Zaino, F. Stefanelli, M. C. Breschi, Gi. Di Colo, 2009. Synthesis, characterization and evaluation of thiolated quaternary ammonium-chitosan conjugates for

enhanced intestinal drug permeation, Eur. J. Pharm. Sci., Volume 38, Issue 2, 10 September 2009, Pages 112-120]. Also, Zambito and Di Colo found that a thiolated quaternary ammonium-chitosan derivative was more effective than the parent non-thiolated derivative in promoting the transcorneal absorption of dexamethasone [Y. Zambito, G. Di Colo, 2010. Thiolated quaternary ammonium–chitosan conjugates for enhanced precorneal retention, transcorneal permeation and intraocular absorption of dexamethasone. Eur. J. Pharm. Biopharm. Volume 75, Issue 2, June 2010, Pages 194-199]. Our present data indicate that thiol groups substituted on the chains of high-MW quaternary ammonium-chitosan conjugates also exert the function of aiding the wound healing process. Relevant data in Table 1 and Figure 2 suggest that the significance of process acceleration is higher for the polymer with higher thiol content. On the other hand, the effects of the thiolated polymers appearing in Figure 3 show poor significance, which can be ascribed, on the basis of the foregoing discussion, to the low MW of rCh compared to Ch. The higher efficacy of the higher-MW chitosan derivatives compared to the lower-MW ones is perhaps ascribable to an ability of the former to establish a co-operative interaction with ECM protein stronger than the latter, because the contributing active sites on the polymer chain are more numerous in the former instance. Nevertheless, the thiols present on the chains of the thiolated low-MW derivatives must still have some wound healing activity, since the rank order of the mean scratch closing rate produced by these polymers, appearing in Figure 3, is the same as that seen in Figure 2 for the significantly active thiolated high-MW derivatives. This order, indeed, corresponds with that of the thiol content listed in Table 1 for the high-MW and low-MW thiolated polymers.

Conclusions

The *in vitro* procedure used in this study has appeared suitable to discriminate between chitosan derivatives for their ability to promote wound healing by acting as a stimulus for fibroblast cell migration. In particular, results revealed that high-MW quaternary ammonium-chitosan conjugates bearing thiol groups on their chains are more effective in promoting cell activity and accelerating

wound healing than the non-thiolated conjugates and the parent non-derivatized Ch. On the other hand, the effects of the corresponding thiolated quaternary ammonium-chitosan conjugates of low MW was statistically insignificant, although some wound healing activity of the thiols carried by these polymers could still be made out. Further studies are needed to understand the molecular basis of the biological response to chitosans and their derivatives…

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