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Article

Investigation of therapeutic effects in the wound healing of chitosan/pGM-CSF complexes

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Granulocyte macrophage colony-stimulating factor (GM-CSF) has been shown to promote the growth, proliferation, and migration of endothelial and keratinocyte cells. Chitosan has been widely used as a biopolymer in wound-healing studies. The aim of this study was to investigate the *in vitro* proliferative effects of chitosan/pGM-CSF complexes as well as the therapeutic role of the complexes in an *in vivo* rat wound model. The effect of complexes on cell proliferation and migration was examined. Wounds were made in Wistar-albino rats, and examined histopathologically. The cell proliferation and migration were increased weight ratio- and time-dependently in HaCaT and NIH-3T3 cell lines. Wound healing was significantly accelerated in rats treated with the complexes. These results showed that the delivery of pGM-CSF using chitosan complexes could play an accelerating role in the cell proliferation, migration, and wound-healing process.

Keywords: Wound healing. Chitosan. pGM-CSF. Complexes.

INTRODUCTION

Wound healing is a dynamic process that can be regulated by many growth factors and cytokines. It is characterized by the regular movement of various cell types into the wound site (Riedel *et al.*, 2006). Acute wound healing consists of a series of co-ordinated overlapping phases that involves hemostasis, inflammation, new tissue formation (proliferation), and remodeling. In chronic wounds, the healing process is impaired, the healing of chronic wounds is difficult and prolongs the time taken for repair (Williamson, Harding, 2004; Li, Chen, Kirsner, 2007).

Multiple cell types, such as fibroblasts, keratinocytes, macrophages, neutrophils, platelets, and endothelial cells, participate in the wound-healing process. In addition, many cytokines and growth factors are released by the various cells to the wound site in the different phases of the healing process (Bryan *et al.*, 2005). These growth factors can be used as pharmacological agents or as a candidate for therapy for enhancing cutaneous wound repair. It is known that granulocyte-macrophage colony-stimulating factor (GM-CSF), a growth factor for granulocyte and macrophage cells, is a pleiotropic and proinflammatory cytokine that facilitates wound healing. Besides, GM-CSF promotes the activation of keratinocytes, fibroblasts and epidermal cells, as well as the movement of dendritic cells and macrophages to the wound site (Enoc, 2007).

Many strategies have been employed to achieve sustained and local delivery of recombinant GM-CSF protein in wound therapy (Samadikuchaksaraei *et al.*, 2019;Yan *et al.*, 2017). Recombinant proteins have to be administered frequently because of their low stability and short biological half-life. However, high and repetitive doses of GM-CSF protein lead to toxicity problems. Another important challenge in the application of exogenous growth factors for wound healing is that they are easily degraded by proteases. Therefore, delivery of genes expressing growth

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factors to wound site could offer an attractive alternative to the injection of recombinant proteins (Eriksson, Vranckx, 2004; Turan, Akbuğa, 2011; Wu *et al*., 2018). However, the application of pDNA in wound therapy is hindered by degradation and poor cellular internalization.

Non-viral gene delivery systems are widely preferred to enhance transfection efficiency and stability. Chitosan is a biopolymer that protects DNA against nuclease degradation by forming polyelectrolyte complexes with DNA (Salva, Turan, Akbuğa, 2011). Chitosan has been reported to have good hemostatic, antibacterial, and bioadhesive properties. These properties of chitosan have attracted attention for wound healing (Tsao *et al*., 2011; Patrulea *et al*., 2015). In our previous study, we investigated the suitability of chitosan as a carrier for the pORF-hGMCSF plasmid encoding the GM-CSF gene. We showed that cell proliferation increased with N/P ratio and time of chitosan/pGM-CSF complexes in HeLa cells (Salva, Turan, Akbuğa, 2011).

Until now, the use of chitosan complexes as a gene delivery system in wound healing has not been reported yet. The present work focuses on two aspects: i) the *in vitro* and *in vivo* investigation of the effects of chitosan/pGM-CSF complexes on wound healing, and ii) the evaluation of chitosan/pGM-CSF complexes as an *in vivo* skin gene delivery system.

MATERIAL AND METHODS

Material

Chitosan (Low MW; 150 kDa, DDA;75-85%) was supplied from Fluka (Germany), DNase I (10.000 U/μg) and MTT cell proliferation kit was from Roche (Germany). pORF-hGMCSF plasmid DNA was supplied from InvivoGen, San Diego, USA. All of the cell culture media and reagents were purchased from Biological Industries (Israel).

Characterization and Preparation of Chitosan/pGM-CSF Complexes

The pORF-hGM-CSF plasmid encodes human GM-CSF. The plasmid DNA is of 3650 bp size and contains SV40 promoter by inserting human GM-CSF with 435 bp (InvivoGen, USA). Plasmid DNA was isolated according to the manufacturer (Roche, Germany). Then, the quantity and quality of the purified plasmid DNA were assessed spectrophotometrically at 260-280 nm and also by electrophoresis in agarose gels.

Chitosan/pGM-CSF complexes were prepared by self-assembly (Salva, Turan, Akbuğa, 2011). Chitosan (1% w/v) was dissolved in 1% acetic acid solution and filtered (0.22 μm) for sterilization. pGM-CSF samples were dissolved in TE (Tris:EDTA, pH 8.0) buffer solution. Complexes were prepared in the different weight ratios (0.05/1-10/1, w/w) by the addition of pGM-CSF solution to a solution of chitosan and left for 60 min in room temperature for forming complexes completely.

For the physical characterization of complexes, their zeta potentials and particle sizes were examined. In addition, different pH evaluation of pH stability of complexes The size and zeta potential of the complexes were measured by Zetasizer 3000 HS (Malvern Instruments, Malvern, UK). The instrument is equipped with both a particle sizer and zetameter unit. Each measurement was done in triplicate.

To test the effect of pH on the chitosan complexes, 10 mM sodium chloride (NaCl) solution were used to adjust the pH level (5.0). The complexes diluted with NaCl were placed in an oven set to 37°C, simulating human body temperature, for 2 h. The effect of the pH on the particle size and zeta potential of the complexes was examined.

The morphological evaluation of complexes was made by transmission electron microscopy (TEM, Jeol, Japan). Complexes for TEM examination were prepared by adding of dilute solution of samples on coated grids and then grids left to dry in air.

MTT Assay

MTT Assay was performed to examine effect to keratinocyte and fibroblast cell proliferation of chitosan complexes containing pGM-CSF. HaCaT and NIH 3T3 cells ($5x10^3$ cells/well) were seeded in 96 well tissue culture plates 24 h prior to the transfection experiments. Prior to transfection, the DMEM was removed and the cells were rinsed once with PBS. The cells were incubated for 48 and 72 h with complexes in different weight ratios

(1/1, 2/1, 5/1, 10/1) in 10% FBS-containing culture medium. Subsequently, MTT (5mg/ml) was added to each well. After 4 h, formed formazon crystals were dissolved in solubilization buffer. Absorption was measured at 570-690 nm using spectrophotometer (Shimadzu, Japan).

In vitro Scratch Wound-healing Assay

Cell migration was measured by scratch wound-healing assay. HaCaT and NIH3T3 cells were seeded and transfected with chitosan/pGM-CSF complexes in 6-well plates (Corning Inc., NY, USA) at the density of 1×10^6 cells/ml. The wound was made through confluent monolayer cells with a 200 µl pipette tip. Wounded monolayers were then washed with PBS, and incubated in DMEM containing 1% FBS. Wound closing was compared between complexes and chitosan-transfected cells and control cells. The width of wound area was monitored with an inverted microscope (Leica, Germany). Photographs of cells were taken at 0, 24, 48, and 72 hours to monitor cell movements (Doan *et al.*, 2014).

In vitro Migration Assay

For migration assay, HaCaT and NIH3T3 cells were seeded and transfected with chitosan/pGM-CSF complexes in 24-well plates (Corning Inc., NY, USA) at the density of 5×10^4 cells per well. HaCaT and NIH3T3 cells were trypsinized and seeded onto 8-µm Transwell inserts (Transwell Coster Corning Inc.). The lower chamber was filled with 500 µl serum-free DMEM and cells were incubated for 24 h at 37ºC (5% CO_2). After 24 h, non-invading cells on the upper surface of membranes were removed by cotton swabs and invading cells on the lower surface of the membranes were fixed with fixative solution and stained with crystal violet. The stained cells were inspected with a microscope and imaged. The experiment was performed in triplicate (Doan *et al.,* 2014).

Formation of Animal Wound Models

Fifty-six Wistar-Albino female rat with a body weight range from 150 to 300 g were used in this study. The hairs on the dorsal side of rats were shaved. Animals were anesthetized and the back area of rats was swabbed with povidone before alcohol application (Turan, Akbuga, 2011). Two full thickness-round wounds were prepared on the upper back of each rats using a 6-mm punch biopsies over an area of approximately 2-3 cm^2. The different (time) intervals after the surgery, the animals were euthanized and biopsy samples from the wounded site were collected. The animal experiments were approved by the Animal Ethical Committee of Marmara University (59.2006.mar).

Treatment of Animal Wounds

100 µg pGM-CSF (10/1) containing chitosan complexes and only chitosan (1%) were applied topically to the wound of each rats. Similar full thickness-round wounds were also prepared as controls without any treatment. Rats were divided equally into two groups. The first experimental group (Group I) received chitosan/pGM-CSF complexes applied topically to the wound and PBS was applied to one of two wounds as control group (wound) on the dorsal surface of the rats. The second experimental group (Group II) received 1% chitosan applied topically to the wound and PBS was applied to one of two wounds as control group. The animals were sacrificed 24., 48. and 72. hours for early phase wound study and 1., 2., 5. and 8. weeks for late phase wound study after treatment. The skin including the wound was removed from each rats for histological examination.

Histopathologic Examination

The removed skins including wound tissue were fixed in a 10% formaldehyde solution. After tissue processing, tissues were embedded in paraffin and sectioned in 3-4 µm thickness and stained with Hematoxylin-Eosin (H&E) and Gomori one step trichrome stains.

Histopathologic evaluation was made considering the criteria modified from those of Lasa *et al.* (1993) and Kirchner *et al.* (2003). According to this, the severity of the inflammatory reaction (acute and chronic inflammation) and neovascularization, the ratio of fibroblast, granulation tissue formation and extent of

epithelization by H&E staining and collagen deposition by Gomori One-Step Trichrome staining were scored (Table I). Two investigators independently scored the histopathology of the wounds.

TABLE I - Histopathologic parameters used to evaluate the effect of chitosan and pGM-CSF on rat wound healing (Lasa *et al.* (1993) and Kirchner *et al.* (2003))

Parameter	Scale of measurement
Acute inflammation	0=absent 1=mild 2=moderate 3=marked
Chronic inflammation	0=absent 1=mild 2=moderate 3=marked
Neovascularization	0=histologically similar to adjacent nonreactive dermis 1= increased vascularity 2=markedly increased vascularity
Epithelization	1= absent 2=minimum; less than half the diameter of the wound 3=completely epithelize, thin layer 4= thicker epithelial layer 5= thick epithelium
Cellular content	1= absent 2= dominant inflammatory cells and a few fibroblast 3= inflammatory cells and fibroblast with less 4=dominant fibroblast 5= a small number of fibroblast in dermis
Granulation tissue	1= absent 2= thick layer of wound edge, no wound center 3=less in wound center, mostly in adipose tissue under the epithelium 4= thicker in the surface of the wound
Collagen deposition	1= absent 2= some collagen fibers 3= more

Statistical Analysis

The data was analyzed for statistical significance using by SPSS 15.0. The results are presented as mean±SEM. Mann-Whitney U test was used to comparing nonparametric variables. A p value of <0.05 was accepted as statistically significant.

RESULTS

Formation and Characterization of Chitosan/pGM-CSF Complexes

We prepared chitosan/pGM-CSF complexes using a simple complexation method. Complexes were formed at different weight ratios by electrostatic interactions between anionic plasmid DNA (phosphate groups) and cationic chitosan (amine groups). The amount of pDNA was kept constant while the amount of chitosan was varied. When the weight ratio was above 0.1/1, the pDNA was completely retained within the gel loading well, as shown in Figure 1.

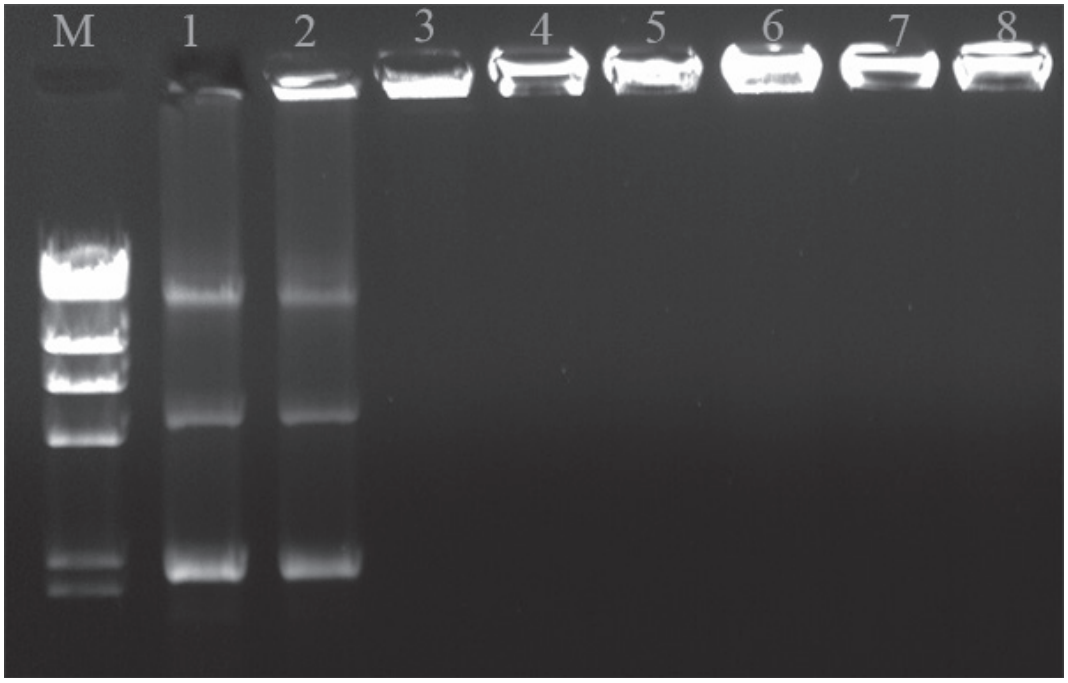

FIGURE 1 - Gel retarding analysis of chitosan/pGM-CSF complexes. λDNA Hind III Marker, Lane 1. Free pGM-CSF; lane 2-8. Chitosan/pDNA complexes at weight ratios of 0.05/1, 0.1/1, 0.5/1, 1/1, 2/1, 5/1, 10/1, respectively.

The positive surface charge and smaller particle size of the complexes facilitated cell entry. In the pH 7.0, the zeta potentials for the chitosan/pGM-CSF complexes (1/1-10/1) ranged from 5.79 to 28.7 mV and the particle sizes ranging from 202 to 482 nm (Table II). The zeta potentials and particle sizes of the complexes increased with increasing amounts of chitosan. In the pH 5.0, the zeta potentials for the chitosan/pGM-CSF complexes (1/1-10/1) ranged from 9.12 to 31.4 mV and the particle sizes ranging from 230 to 514 nm (Table II). The zeta potentials and particle sizes of the complexes increased with decreasing of medium pH.

The morphology of complexes was controlled with TEM (Figure 2). Chitosan/pGM-CSF complexes showed spherical and branched shape.

TABLE II - Zeta potential and particle size values of chitosan/pGM-CSF complexes at the different pH values

Formulations	Zeta Potential (mV±SD)	Particle Size (nm±SD)
Free pGM-CSF	-13.2±3.2	
Chitosan	35 ± 2.83	
pH 7.4		
Chitosan/pGM-CSF 1/1	5.79 ± 1.00	202 ± 4.31
Chitosan/pGM-CSF 2/1	10.1 ± 0.16	262 ± 21.0
Chitosan/pGM-CSF 5/1	15.0 ± 0.60	333 ± 2.47
Chitosan/pGM-CSF 10/1	28.7 ± 0.71	481.6 ± 13.8
pH 5.0		
Chitosan/pGM-CSF 1/1	9.12 ± 1.41	230 ± 12.11
Chitosan/pGM-CSF 2/1	12.6 ± 1.41	259 ± 16.32
Chitosan/pGM-CSF 5/1	28.3 ± 0.64	409 ± 23.85
Chitosan/pGM-CSF 10/1	31.4 ± 1.76	514 ± 12.3

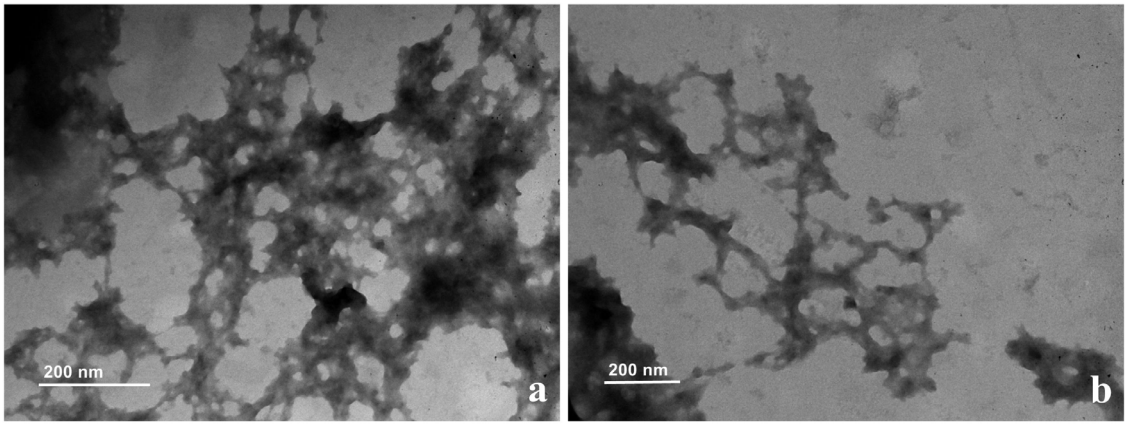

FIGURE 2 - TEM images of chitosan/pGM-CSF complexes (10/1). a. 75.000× magnification, b.100.000× magnification.

Effects of Complexes on Keratinocyte and Fibroblast Cell Proliferation

The effects of the chitosan/pGM-CSF complexes on the proliferation of HaCaT keratinocyte and NIH-3T3 fibroblast cell lines were determined by MTT assay. Changes in GM-CSF gene expression-dependent cell proliferation during the experiment are shown in Figure 3. Treatment with the chitosan/pGM-CSF complexes led to higher cell proliferation in both cell lines than free chitosan. The complexes accelerated the proliferation of keratinocyte and fibroblast cells with increasing weight ratio (1/1-10/1) and increasing time of exposure (48 and 72 hrs). After 72 h of culturing, the absorbance value was higher than that at 48 h. The cell proliferation was significantly increased by the 10/1 ratio of complexes ($p<0,05$).

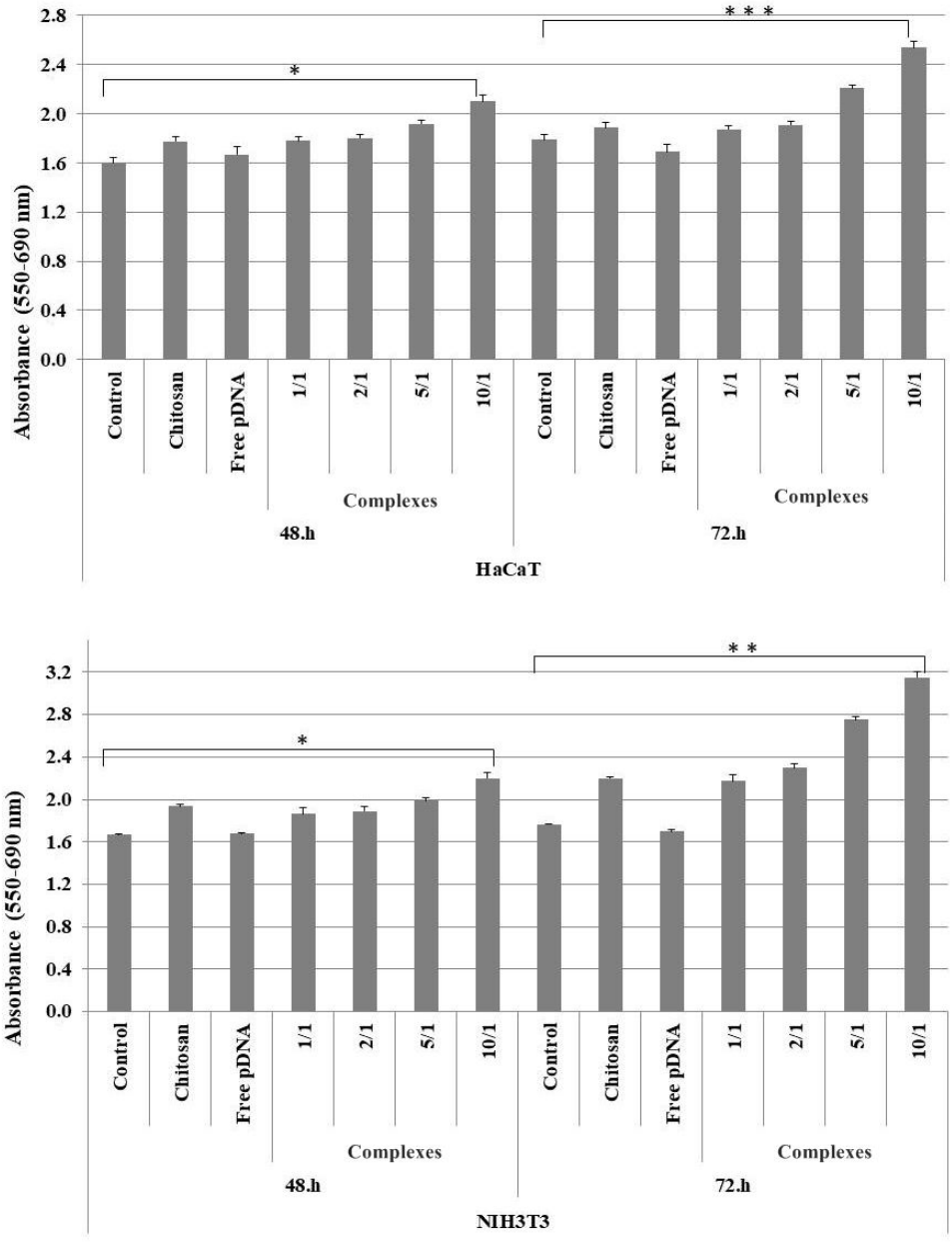

FIGURE 3 - MTT analysis of effect to cell proliferation of chitosan/pGM-CSF complexes in HaCaT and NIH3T3 cells at 48. and 72. hrs.

Effect on Cell Migration Ability of Complexes

The migration phase in wound healing is important for restoration of damaged tissue by migration of fibroblasts and epithelial cells to the injured area (Grimstad *et al.*, 2011). Since the greatest cell proliferation in the MTT study was seen with the 5/1 and 10/1 ratios of complexes, these ratios were used for the cell migration study. The wound healing assay was used to evaluate the migration ability of HaCaT and NIH3T3 cells after transfection of chitosan/pGM-CSF complexes (5/1 and 10/1 weight ratios). The cell monolayer was scratched and cells migrating to the wound area were monitored at different time points. As illustrated in Figures 4(a) and 4(b), compared with cells in the control group, the cells transfected with the chitosan/pGM-CSF complexes showed a narrow wound area 24 hours after wound generation, indicating a defect in the migration. Cells treated with pGM-CSF exhibited an increase in wound-healing ability compared to the untreated control. The scratches in the wells transfected with the complexes were completely closed after 72 hrs in both cell lines.

Besides the scratch assay, we investigated cell migration using uncoated inserts on transwell chambers. After incubation for 24 h, the migration ability in the cells transfected with complexes was significantly increased (Figure 5).

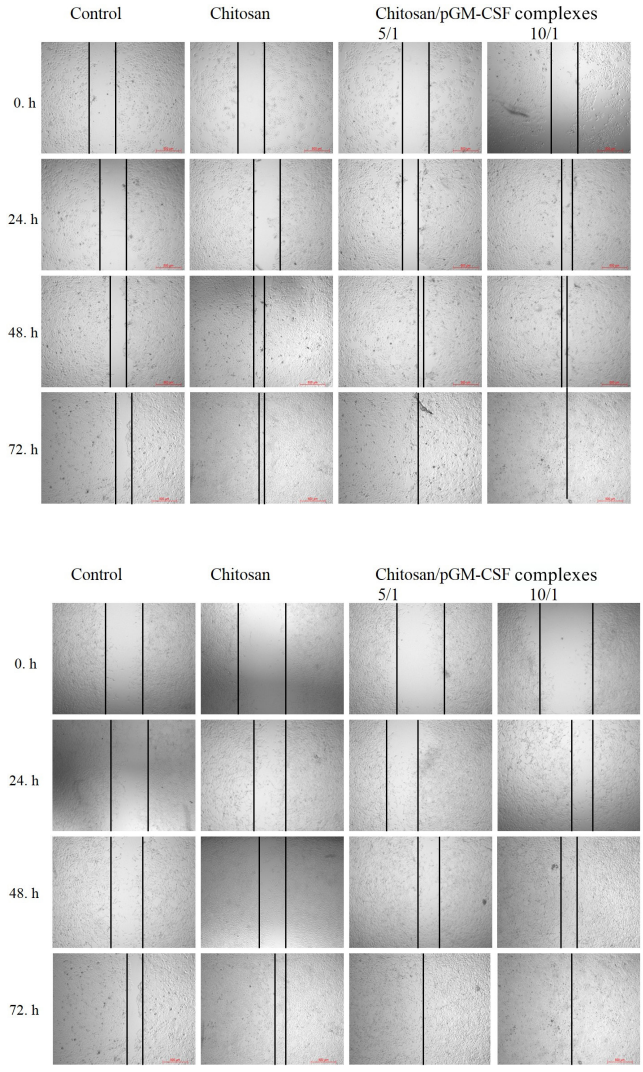

FIGURE 4 - Scratch assay in **a)** HaCaT cells and **b)** NIH3T3 cells.

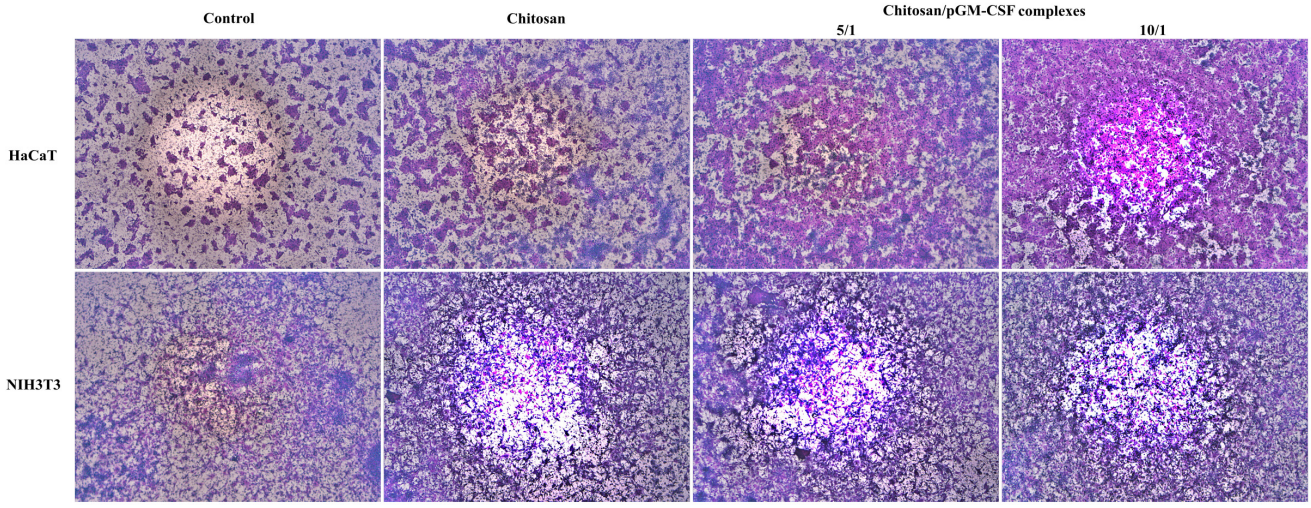

FIGURE 5 - In vitro migration assay in HaCaT and NIH3T3 cell lines.

Histopathologic Findings

Wound healing comprises several phases including inflammatory, proliferative and remodeling. Cellular and molecular processes play a key role in the regulation of all these overlapping phases. The interaction of cells in the dermis and epidermis is required for wound healing. The early phase of wound healing is characterized by migration of granulocytes, macrophages, neutrophils, keratinocytes, endothelial cells and fibroblasts to the wound site (Grimstad *et al.,* 2011).

In this study, we investigated the effects of the chitosan/pGM-CSF complexes (10/1) and chitosan on the wound-healing process in the early (24, 48, and 72 hrs) and late stages (1, 2, 5 and 8 weeks). The effect of chitosan/pGM-CSF complexes on wound healing were evaluated based on ‘’acute and chronic inflammation, neovascularization, epithelization, cellular content, granulation tissue, and collagen deposition’’ criteria histopathologically (Lasa *et al.*, 1993; Kirchner *et al.*, 2003).

Effect on Inflammation of Complexes

The inflammatory reaction was examined in two stages: acute and chronic. In the acute inflammatory reactions, inflammatory cells were mainly composed of granulocytes in the early stages of healing. In the chronic inflammatory reactions, typical cells are macrophages and lymphocytes in the late stages of healing (Greaves *et al.*, 2013). The significantly increased infiltration of inflammatory cells and neutrophil migration were observed in chitosan/pGM-CSF complexes and free chitosan-treated wounds at 24 hrs compared with the control. While the neutrophil migration was induced with chitosan/pGM-CSF complexes at the early stage, it was substantially decreased at the late stage (Figure 6). After 24 hrs, inflammation in the complex-treated group was markedly reduced. At day 7, the difference between control and treatment groups became even more marked (Figures 7(a-b)).

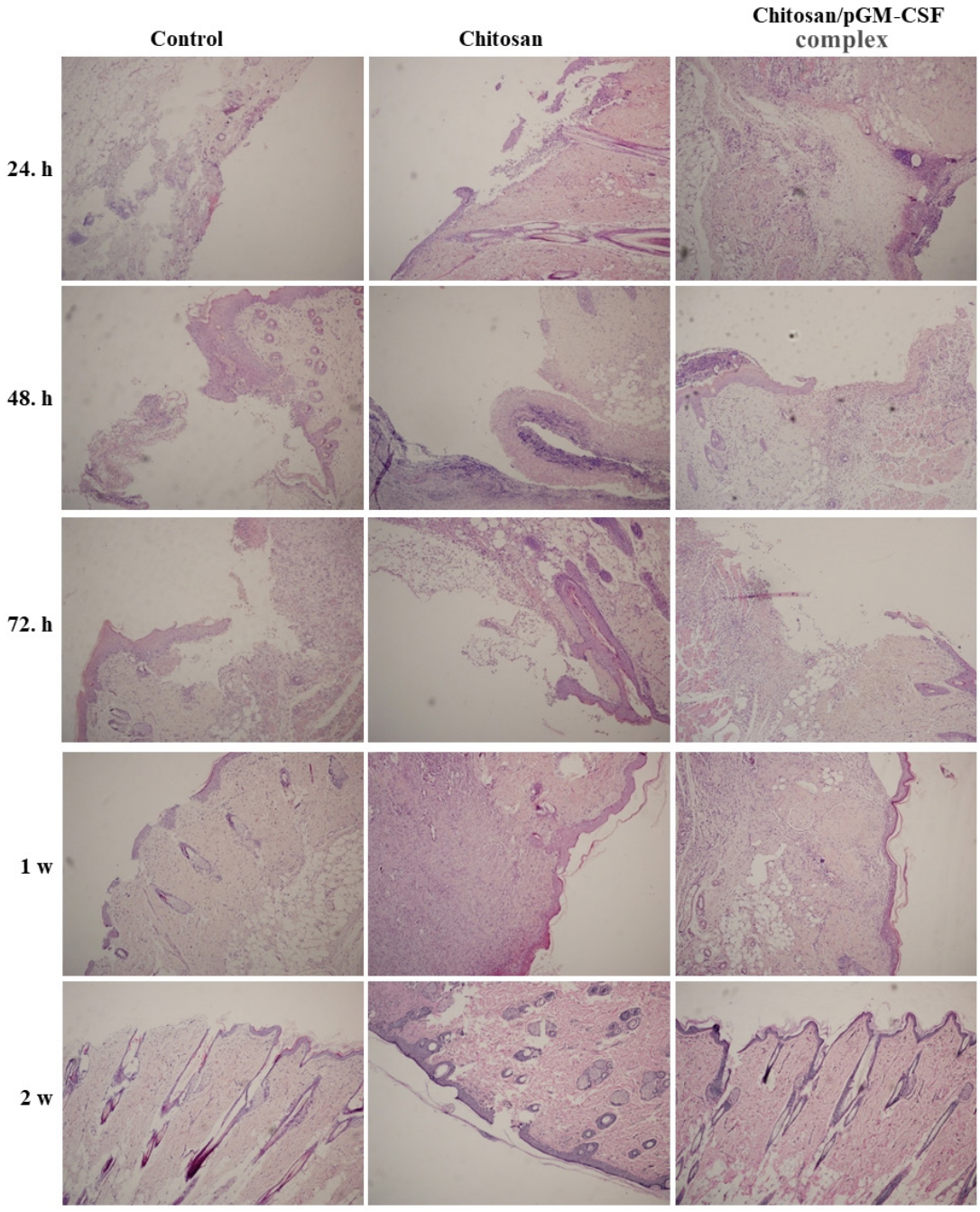

FIGURE 6 - Wound healing in early and late stages.

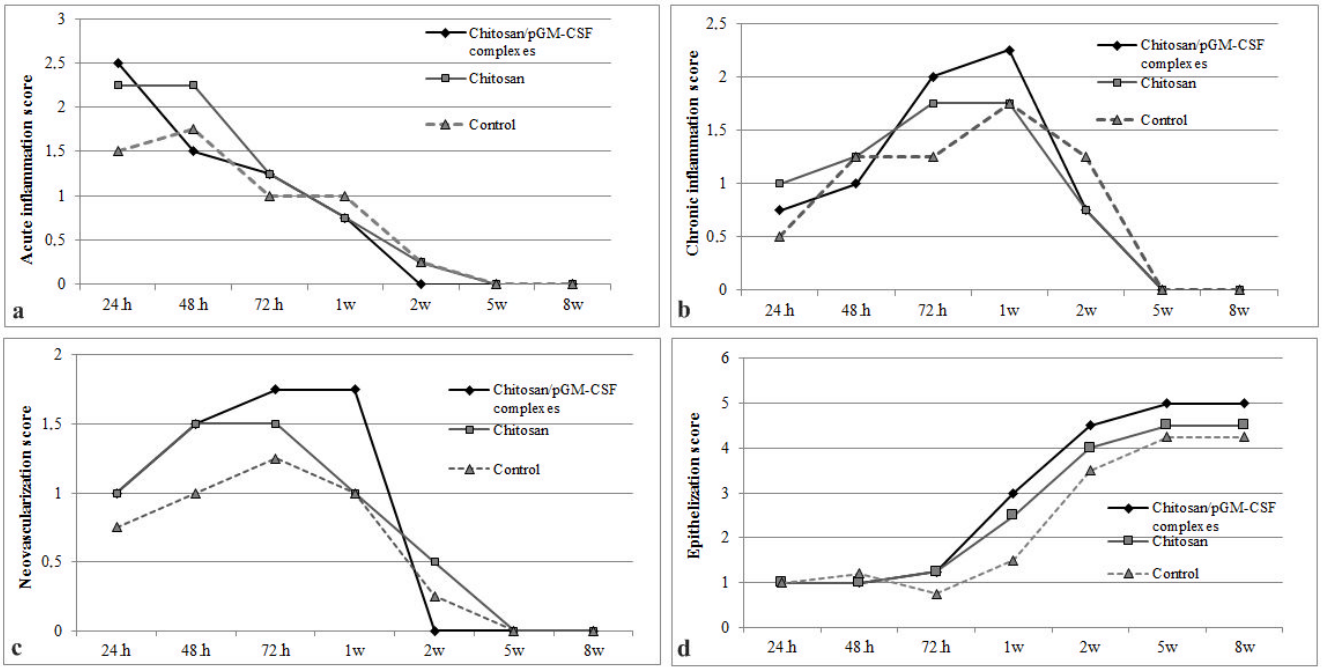

FIGURE 7 - Changes in (a-b) acute and chronic inflammation scores, (c) neovascularization score, (d) epithelization score depending on time.

Effect on Proliferation and Neovascularization of Complexes

The proliferative phase is characterized by granulation tissue formation, neovascularization, epithelization, collagen deposition and wound contraction (Minagawa *et al.*, 2007). Figure 7(c) shows the effects of the complexes on neovascularization. Neovascularization induced by complexes reached a maximum level between days 3 and 7. In the late stage, the neovascularization score was markedly reduced in the complexes-treated group compared to the other groups. Chitosan complexes containing pGM-CSF had a substantial effect in inducing vascularization (Figures 6 and 7(c)). Several reports have demonstrated that neovascularization of wounds is strongly enhanced by GM-CSF expression (Boateng *et al.*, 2008; Mann *et al.*, 2001).

Effect on Reepithelialization of Complexes

Reepithelialization begins within hours post-wounding and keratinocyte migration occurs (Singh, Shitiz, Singh, 2017). Figure 7(d) illustrates the differences in the rates of reepithelialization among the treatment groups. The highest epithelization scores were found in the complexes-treated group in the late stage. In the early stage, epithelization of the wounds was not observed. Epithelization in the chitosan and complex groups started between 3 and 7 days post-wounding. The closure of the wounded area was completed in at 14 d post-wounding. Mann *et al.* (2001) reported that GM-CSF expression accelerated reepithelialization of full-thickness skin wounds.

Effect on Cellular Content of Complexes

The levels of fibroblasts, inflammatory cells, and endothelial cells increased progressively during proliferative phase. Figure 8(a) shows that, comparing all the groups, inflammatory cells were more dominant in the early stage and macrophages and fibroblasts predominated in the late stage. During the wound-healing process, the cellular content was markedly increased in the free chitosan and chitosan/pGM-CSF complexes groups compared to the control group. In the transition from the inflammation phase to the proliferation phase of the healing process, some of the inflammatory cells

were replaced by fibroblasts. The highest level of fibroblast proliferation was observed at 7 days in the chitosan/pGM-CSF complex group. Cellular content also markedly decreased at 14 days in all groups (Figure 6). Minagawa *et al.* (2007) reported a relationship between the amino residues of chitosan and fibroblast activation. Many activated fibroblasts were observed around the wound region when chitosan was applied. The higher the degree of deacetylation of chitosan, the stronger the break strength and the more fibroblasts are activated.

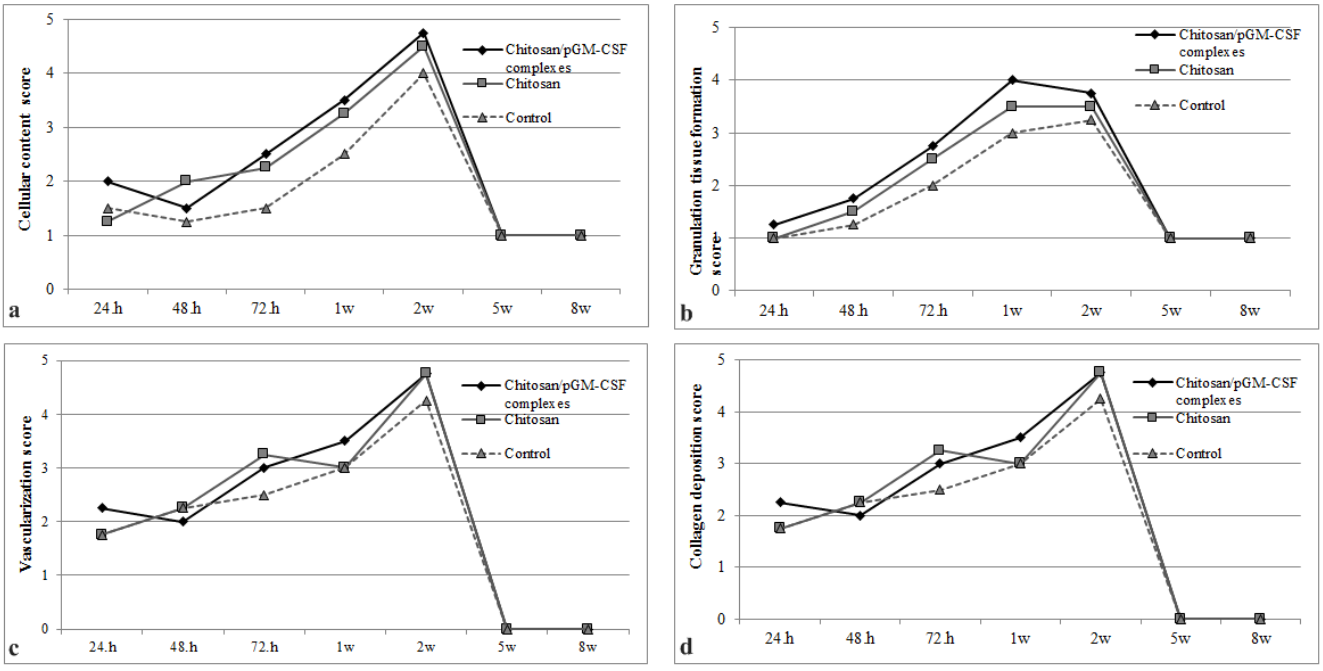

FIGURE 8 - Changes in (a) cellular content score, (b) granulation tissue formation score (c) vascularization score, (d) collagen deposition score depending on time.

Effect on Granulation Tissue Formation of Complexes

The proliferation and migration of fibroblasts, macrophages, and polymorphonuclear leucocytes (PMNLs) enhance the levels of macrophage secretory products, plasminogen activator and lymphokines in the wound area (Grimstad *et al.*, 2011). Thus, the formation of highly vascular new granulation tissue begins and continues until the wound is covered. Table I details the criteria relating to localization of granulation tissue on the wound. The formation of granulation tissue and vascularization were accelerated in the complex group (Figures 6 and 8(b,c)). The formation of granulation tissue is important for the closure of the wound because fills defect areas. Several studies have demonstrated that chitosan accelerates the granulation and remodeling stages of wound healing (Minagawa *et al.*, 2007; Singh, Shitiz, Singh, 2017).

Effect on Collagen Synthesis of Complexes

In the remodeling or maturation phase of wound healing, production and remodeling of collagen, as well as the differentiation of fibroblasts into myofibroblasts for wound contraction and scar formation occur to increase the wound's tensile strength (Değim, 2008). In wound healing, fibroblasts that secrete new collagen fibers migrate to the wound area at 5 or 7 days and fibroblast proliferation and collagen synthesis continues for up to 2 weeks (Boateng *et al.*, 2008). Gomori's trichrome staining showed the collagen remodeling and maturation. Collagen synthesis in the wound area is crucial for the

remodeling of tissue as well as tissue tensile strength (Li *et al*., 2012). Collagen deposition in the free chitosan and complex groups was increased in the early and late phases of wound healing as shown in Figure 8(d). The results demonstrated that the mature collagen area of the wound treated with complexes was much bigger than that of the control. Collagen deposition was stimulated by the chitosan/pGM-CSF complexes.

DISCUSSION

Growth factors, chemokines, and proinflammatory cytokines are important mediator molecules for acute wound healing (Barrientos *et al*., 2014; Werner, Grose, 2003). GM-CSF is a pleiotropic cytokine that stimulates proliferation and migration of endothelial cells and keratinocytes. The production and function of PMNL are also increased by GM-CSF. In addition, GM-CSF promotes the healing of difficult or non-healing wounds. Skin wounds with treated GM-CSF showed enhanced healing by neovascularization, reepithelialization, cell migration and proliferation with better healing rates (Barrientos *et al*., 2014; Werner, Grose, 2003).

The synthesis of extracellular matrix (ECM) components is required for wound repair. Chitosan is present in the ECM and comprises poly-*N*-acetylglucosamine with beta (1-4) glycosidic linkage and N-acetyl groups (Ribeiro *et al*., 2009). Indeed, it resembles mammalian glycosaminoglycans (GAGs) structurally. Chitosan can bind to mucosal surfaces owing to its bioadhesive properties. The high deacetylation degree of chitosan enhances the electrostatic interactions of cationic amine groups with anionic glycosaminoglycans, proteoglycans, and other negatively charged molecules in cell membranes. Chitosan's degradation products are biocompatible and have no cytotoxic effects. In addition, chitosan is a procoagulant that can arrest bleeding and exhibits anti-microbial activity (Ampuero *et al*., 2015; Chang *et al*., 2013). These properties of chitosan allow for its use as an effective polymer for drug delivery in wound healing. The delivery by using polymeric systems of the desired growth factor to the wound site is an effective wound-healing strategy. Because of the short half-life times, high doses, and costs of recombinant growth factor proteins, the delivery of growth factor genes or genetically modified cells using polymeric systems could be preferred (Eming, Krieg, Davidson, 2007; Gauglitz, Jeschke, 2011; Wu *et al*., 2018).

In the present work, we demonstrated the ability of pGM-CSF-containing chitosan complexes to promote healing in the early and late wound phases and increase the proliferation and migration properties of fibroblast and keratinocyte cells. We had previously demonstrated chitosan complexes to be an efficient system for the delivery and release of pGM-CSF to cells (Turan, Akbuğa, 2011). For successful non-viral gene therapy, a balance between DNA binding of the polymer, DNA protection and DNA unpacking inside cell is important (Agirre *et al*., 2014). The complexes prepared with low molecular weight chitosan are less stable, but release of the DNA inside the cells is higher, than those with prepared high molecular weight chitosan (Lee *et al.* 2001**;** Duceppe, Tabrizian, 2009). Köping-Höggård *et al.* (2004) suggested that chitosan polyplexes protected pDNA against enzyme degradation and provided efficiently release and delivery of pDNA. The appropriate positive charge, particle size, and morphology of the complexes could facilitate cell entry (Lavertu *et al*., 2006). The pH of the skin has been reported to be in the range of 5.4 to 5.9. The aqueous solutions in the pH 5 and 7 did not cause irritation when applied to the skin (Paudel *et al*., 2010). Lavertu *et al.* (2006) suggested that zeta potential value of complexes increases in an acidified medium and the aggregation of complexes decreases with increase of electrostatic repulsion. In this situation, chitosan complexes in slightly acidic medium are more stable and more efficient for transfection to cell. In our study, the effect of pH on the particle size and zeta potential was evaluated. It shown that as the pH of the medium decreases, the zeta potential and particle size of complexes increased.

The effects of chitosan/pGM-CSF complexes on cell proliferation and migration were evaluated by MTT and wound-healing assays. When compared with the control group, the greatest cell proliferation and migration were observed with the 10/1 complexes ratio for both cell lines. GM-CSF induces the many growth factors or cytokines in a paracrine manner, which strongly stimulate keratinocyte and fibroblast proliferation *in vitro* and are upregulated in

wound healing (Heo *et al.*, 2012). These results suggest that pGM-CSF containing chitosan complexes attenuated the wound healing potential.

It has been known for a long time that chitosan accelerates wound healing. The chitosan is degraded in the wound area, N-acetyl-β-D-glucosamine is released and absorbed from the wound. It is reported that chitosan provides remodelling in wound healing by increasing the collagenase acitivity especially synthesized by fibroblasts (Minagawa *et al.,* 2007; Miguel, Moreira, Correia, 2019). Samadikuchaksaraei *et al.* (2019) showed that applying G-CSF to the wound site increases collagenesis and fibroblast infiltration. In our study, remodelling and regeneration occured in wounds treated with chitosan and chitosan/pGM-CSF complex groups, as compared with the control.

CONCLUSION

In our i*n vivo* study, H&E and Gomori's trichrome staining results demonstrated that chitosan/pGM-CSF complexes accelerated wound healing in the early and late phases. According to our results, chitosan complexes are suitable for use as a delivery system for the GM-CSF gene in wound-healing therapy.

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