Vitamin C-Loaded Albumin Nanoparticles Treatment and Its effect on Collagen I and III and miR-133 Gene Expression in Mice

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Abstract

Wound healing is a complex biological process in which many molecules, including microRNA molecules, play an essential role in its regulation. It is well-established that reducing miR-155 expression can accelerate wound healing. This study investigated the effect of using nanoparticles loaded with vitamin C on miR-133, collagen I, and III expressions. In this study, first, nanoparticles of albumin protein were produced and then loaded with vitamin C. 3T3 mouse fibroblast cells were affected by these nanoparticles, and cell behavior was investigated to evaluate the toxicity and appropriate doses. In addition, the expression of collagen I and III genes was studied. The results showed that nanoparticles containing vitamin C in 20 µg/ml concentration had a positive effect on collagen I and III expressions compared to the control group. Moreover, we observed a decrease in the expression of miR-133 in comparison to the control group. Therefore, according to the results of this study, it can be argued that nanoparticles containing vitamin C can significantly decrease the expression of the miR-133 gene and lead to collagen I and III gene overexpression in fibroblasts cells, which is directly effective in wound healing.

Introduction

Wound healing, in which the skin repairs itself in response to damage, is a complex process. It involved several stages: homeostasis, inflammation, proliferation, and finally, the regenerative phase (Rezaie et al., 2019). These stages occur in continuous biochemical events and eventually leads to the repair of damaged tissue. The speed of wound healing depends on several local and systemic factors, the state of inflammation and the levels of growth factors. Therefore, regulation of inflammation factors and candidate gene expression in wound healing is essential (Kirsner and Eaglstein, 1993).

Fibroblasts are one of the most important cells involved in wound healing. They are one of the active cells in almost all stages and advancement of this process. Fibroblasts can invade the fibrin clot, destroy it and pave the way for the other repair cells to migrate to the wound space (Xie et al., 2008). They produce and secrete extracellular matrix components and interact with them (Frantz et al., 2010).

The extracellular matrix is another critical factor in the proper advancement of wound healing and provides a scaffold and physical support for the formation of new tissues and acts as a storage repository for restorative cytokines (Frantz et al., 2010). Therefore, the proliferation and activity of fibroblast cells regulate the production and secretion of extracellular matrix components, such as collagen I and III, for the proper and timely healing of wounds. The role of collagen I and III in optimizing wound healing is well-known, so it is essential to know how they are expressed and formed and the related substrates or drugs involved with them (Klinge et al., 2000).

Vitamin C can be found in its large amounts in healthy skin, responsible for stimulating collagen synthesis and protection against light damage caused by UV radiation. During the proliferative phase of wound healing, fibroblasts produce collagen fibers using divalent ions such as iron and copper and vitamins such as vitamin C (Lanman and Ingalls, 1937).

MicroRNAs (miRNAs or miRs) are a subset of non-coding RNAs containing 18-22 nucleotides (Teymoori et al., 2017) that are evolutionarily conserved and have pivotal roles in the progression

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of the various biological process, including wound healing (Sanjari et al., 2015). The TGFb1 gene is considered as one of the principal targets of miR-133a (Roderburg et al., 2013).

Nanotechnology is a multidisciplinary science including various aspects of research and technology. Nanoparticles are some kinds of metal substances with a size range of 1-100 nm and are in the form of blocks made of nanotechnology. Metal nanoparticles such as gold, silver, and platinum have gained considerable attention in recent years due to their helpful basis and technology. These nanoparticles have the unique catalytic, electronic and optical properties of metal particles. Many methods have been developed for the synthesis of nanoparticles in recent years, such as physical, chemical, and biological methods (Saxena et al., 2020).

In this study, vitamin C-loaded human serum albumin (HAS) nanoparticles were made, fibroblast cells were affected in different group treatments, and then the expression of various genes, including miR-133 and collagen I and III had been examined.

Materials and Methods

Nanoparticle Production

As fully explained in the previous study (Shojania et al., 2019): HSA nanoparticles (Octalbin 20%) are a sterile protein solution containing at least 96% human albumin. In order to make albumin nanoparticles, the following steps were performed: 250 µl of Octalbin solution (equivalent to 50 mg of HSA) was added to 750 µl of distilled water, and adjusted the pH of the solution to 7.4. Then Tween 80 (2% v / v) was added to the solution and stirred for 30 minutes at 500 rpm (round per minute), followed by adding 4 ml of ethanol (dropwise) to the stirring suspension. Then 12 µl of 8% aqueous glutaraldehyde solution was added to the suspension to concentrate the nanoparticles. In the next step, nanoparticle suspension was stirred for another 500 rpm for 24 hours at room temperature. Finally, nanoparticle suspension was centrifuged at 500 rpm for 15 minutes at 4 °C to remove any impurities. Vitamin C-loaded albumin nanoparticles were prepared by the nanoprecipitation method. Briefly, 250 µl of Octalbin solution (50 mg of HSA) was added to 750 µl of distilled water, and pH was adjusted to 7.4. Tween 80 (2% v/v) was added to the solution and stirred at 500 rpm for 30 min. Then, 25 mg of vitamin C was added to the solution, and 4 ml of ethanol was gradually dropped into it. After the desolvation process, 20 µl of 8% glutaraldehyde

aqueous solution was added to promote particle condensation under 500 rpm for 5 h. Nano suspension was centrifuged at 5000 rpm for 15 min at 4°C and washed with PBS for further analysis (All chemicals were purchased from Sigma-Aldrich) Further purification of nanoparticles was performed by the dialysis method. For nanoparticle characterization particle size and zeta potential were measured using the size and zeta analyzer (Beckman Coulter) also Nanoparticles morphology was investigated by using an EM3200 scanning electron microscope. Freeze dried samples were prepared and used for electron microscopy.

Cell

In this study, 3T3 fibroblast cells were provided from Sabzevar University of Medical Sciences and were cultured in T25 flasks (1.2×10^6). They were grown in DMEM medium (Gibco) with high glucose supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/Streptomycin antibiotic (Sigma-Aldrich). The cells were maintained at 37 ° C with 5% CO2 and 95% air (memmert incubator). They were passaged after thawing and followed in appropriate confluency.

Cell Viability Assay

Cells were cultured in 96-well plates for 24- and 48-hours incubation periods for cell cytotoxicity assessment. After 24 hours, the previous medium was exchanged, and 5, 10, 20, 30, 40 µg/ml of nanoparticle concentrations were added in 5 replications. The control group was considered separately for each group of concentrations, and PBS was used instead of the same amount of nanoparticle solutions. MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) according to the kit (Biotium) instructions was performed after 24- and 48-hours treatments; the supernatant was removed, and 100 µl of RPMI 1640 solution containing 10 µl of 0.5 mg/ml MTT solution was added to each well. The plate was incubated at 37 ° C for 4 h. After formazan crystals formation, the previous medium was removed, and 100 µl of DMSO solution was added. After several pipetting, the plates were incubated for 10 minutes, and then the light absorption at 570 nm was read by the plate reader.

Trypan Blue Staining

Trypan blue was used to study the ratio of living cells to dead cells in cell cultures. Since living cells are resistant to the penetration of this substance into the cell membrane, dead cells were shown in blue

color. All concentrations and replications were performed as MTT assay and following the protocol.

Real-time PCR

Exiqon provided the primers applied in this study. RNA extraction and cDNA synthesis, by oligo dT primers, were carried out according to the company's protocols and kits (TRIpure reagents and TaKaRa cDNA synthesis kit, respectively). SYBR Green was used to perform real-time PCR (Rotor- Gene 3000). The primer efficiency was calculated, and then real-time PCR analysis was performed for the samples (Takapozist SYBRgreen). All primers and sequences used are listed in Table 1.

Table 1. The list of primers used for real-time PCR.

| primer	Sequence	NCBI Reference Sequence
Col I	F: ACATGTTCAGCTTTGTGGACC	NM_007742.4
	R: GGTTTCCACGTCTCACCATT	
Col III	F: CTGTAACATGGAAACTGGGGAAA	NM_009930
	R: CCATAGCTGAACTGAAAACCACC	
b-actin	F: TTCTTTGCAGCTCCTTCGTT	BC138614.1
	R: ATGGAGGGGAATACAGCCC	

Results

Nanoparticle Synthesis

In this study, the exact synthesized nanoparticles for the previous article were used, and all confirmations (including measurement of zeta potential and defining size using electron microscope photography) are available in the previous report (Shojania et al., 2019).

Cell Viability Assays

In this study, two methods (MTT assay and trypan blue staining) were used to study cell survival and growth behavior. The effect of HSA nanoparticles carrying vitamin C on viability, growth, and proliferation of 3T3 fibroblast cells at 24- and 48-hours treatments was evaluated by MTT assay. The results showed that vitamin C-carrying HSA nanoparticles in 20 µg/ml concentration had the most significant stimulatory effect on the growth of 3T3 fibroblast cells compared to the control group both in 24 and 48 hours after treatments ($P < 0.05$) (Figure 1). In addition, the trypan blue staining method also provided similar results (Figure 2).
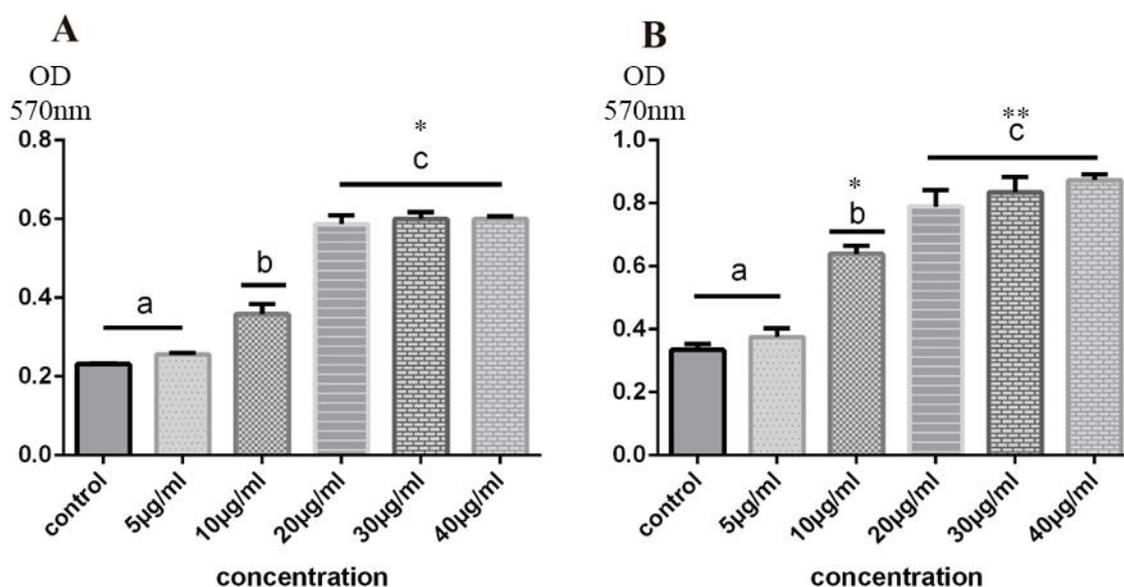
Figure 1. MTT assay test of vitamin C-loaded HSA nanoparticles on 3T3 fibroblast cells after 24 (A) and 48 h (B). 20 µg/ml of vitamin C-loaded HSA nanoparticles induced 3T3 fibroblast cells growth and proliferation after 24 and 48 h (P ≤ 0.05).The vertical axis represents the optical density (570 nm), and the horizontal axis represents the nanoparticle concentration.
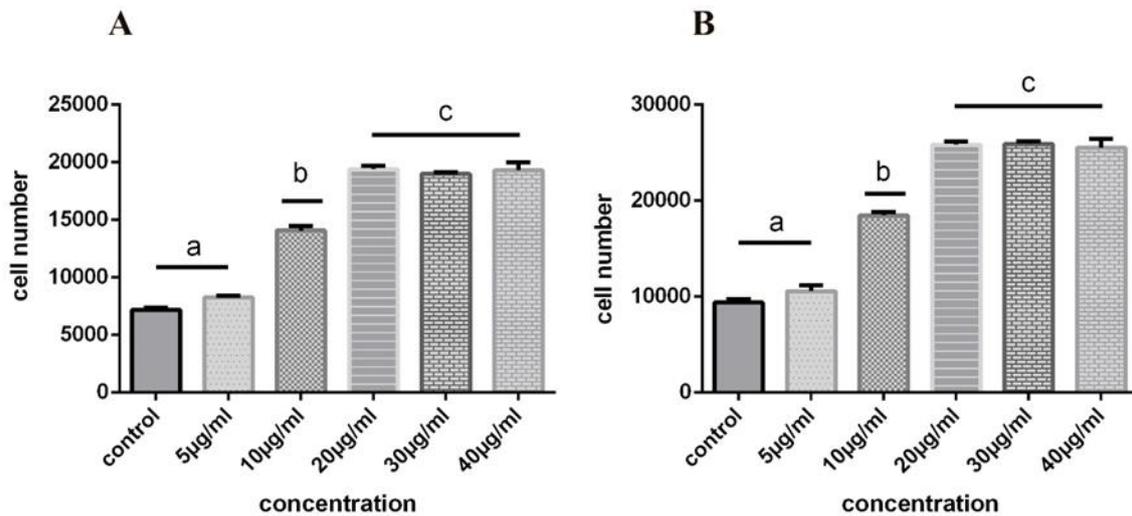

Figure 2. Trypan blue staining test of cell viability of vitamin C-loaded HSA nanoparticles on 3T3 fibroblast cells after 24 (A) and 48 h (B). 20 µg/ml of vitamin C-loaded HSA nanoparticles induced 3T3 fibroblast cells growth and proliferation after 24 and 48 h (P ≤ 0.05). The vertical axis represents the number of cells, and the horizontal axis represents the concentration of nanoparticles.

Gene Expression

RNA extraction and cDNA synthesis were performed from the treated and untreated fibroblast cells. The expression of *miR-133*, type I and type III collagen genes were assessed. As shown in Figure 3, a decrease in the expression of miR-133 and an increase in expression of type I and III collagen were observed in cells treated with vitamin C–loaded HSA nanoparticles.
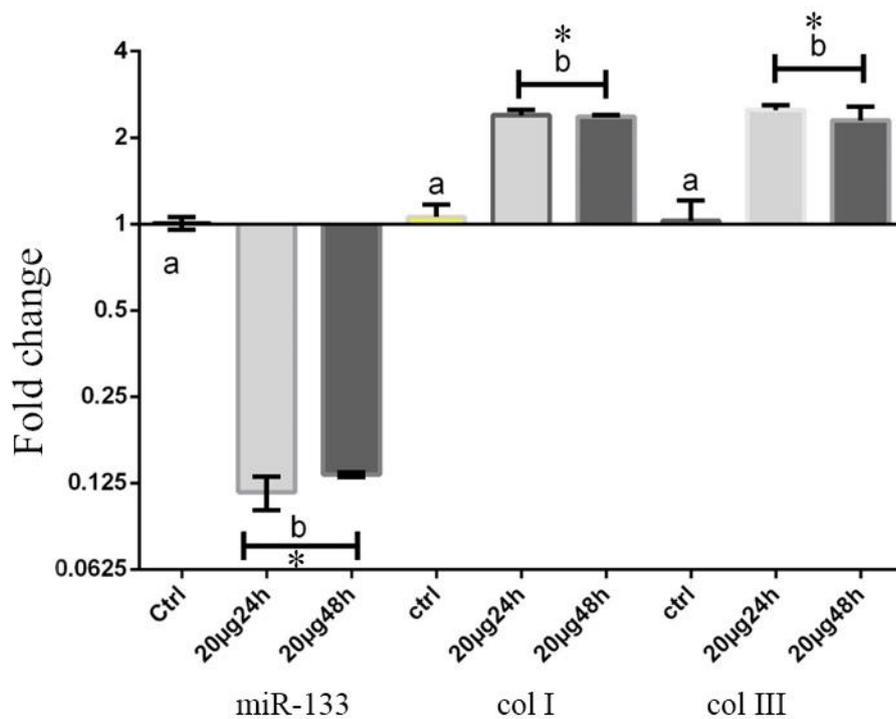
Figure 3. miR-133, type I and type III collagen expression in control and vitamin C-loaded human serum albumin (HSA) nanoparticles treated groups. The level of miR-133 in vitamin C-loaded nanoparticles treated groups was significantly decreased after 24 and 48 h compared with control groups. On the other hand, types I and III collagen level was significantly increased (P < 0.05) compared to the control group.

Discussion

Many factors are involved in wound healing. One of these factors is the proper formation of collagen fibers in damaged tissue, which is done by fibroblast cells. While any defect in collagen synthesis can cause problems, finding ways to properly manage collagen production in the wound bed can accelerate the wound healing process. Today, with the significant growth of nanotechnology, producing efficient nanoparticles in the field of wound healing can be introduced as a promising option (Saxena et al., 2020).

This study sought to investigate the effect of human serum albumin (HSA) nanoparticles containing vitamin C on collagen expression in fibroblast cells. For this purpose, we first studied the effective concentration of this nanoparticle on cells and then we examined expression changes of col I, col III, and miR-133, using the real-time PCR method.

Collagen synthesis by fibroblast cells is directly involved in wound healing. This extracellular matrix can be introduced as a suitable substrate for the placement of other cells, so any factor that enhances collagen gene expression can be considered in this process (Clore et al., 1979).

Based on the literature, vitamin C plays a major role in collagen synthesis by inhibiting the production of proline hydroxyl in treating of skin wounds (Lanman and Ingalls, 1937). This study examined the toxicity of nanoparticles and their biocompatibility on fibroblast cells, the main target. The results showed that none of them are toxic in the range of tested concentrations. Maximum cell growth was observed at 20 µg/ml. This concentration was chosen for further experiments. Not only vitamin C-loaded HSA nanoparticles did not show any toxicity in the evaluated dosage, but also, they could increase mouse fibroblast cell growth.

In addition, we also sought to investigate miR-133 expression changes in response to nanoparticles. Studies have shown that miR-133 can control collagen synthesis well through the TGF-β pathway (Duan et al., 2015). In the previous research, we showed the relationship between miR-155 and TGF-β as well (Shojania et al., 2019). According to our findings in these reports, there was an exciting point in the relationship between miR-133, miR-155, and wound healing. By reducing collagen synthesis, both of them showed an inhibitory role. So, treatment with nanoparticles containing vitamin C can remove this inhibition and accelerate wound healing. The effect of miR-133 through TGF-β-dependent depression of collagen synthesis has been well described, especially during liver fibrosis (Roderburg et al., 2013). The inhibitory role of the miR-133 molecule on collagen synthesis is defined in the present study, and nanoparticle treatment could significantly reduce the expression of miR-133 and increase col I, col III molecules simultaneously.

Drug control of wound healing mechanisms is of great importance. Any irregularity in this process leads to chronic conditions and/or prolongation of treatment. What has been done in this study can be a good candidate for inducing collagen expression by fibroblasts at the wound site. In addition, we did similar experiments in the previous study (Shojania et al., 2019), and parallel with this study, miR-133, in coordination with miR-155, showed precisely the pattern toward faster healing of the wound.

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