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Gene therapy based on interleukin-12 loaded chitosan nanoparticles in a mouse model of fibrosarcoma

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ABSTRACT

Objective(s): Interleukin-12 (IL-12) as a cytokine has been proved to have a critical role in stimulating the immune system and has been used as immunotherapeutic agents in cancer gene therapy. Chitosan as a polymer, with high ability of binding to nucleic acids is a good candidate for gene delivery since it is biodegradable, biocompatible and non-allergenic polysaccharide. The objective of the present study was to investigate the effects of cells transfected with IL-12 loaded chitosan nanoparticles on the regression of fibrosarcoma tumor cells (WEHI-164) *in vivo*.

Materials and Methods: WEHI-164 tumor cells were transfected with IL-12 loaded chitosan nanoparticles and then were injected subcutaneously to inoculate tumor in BALB/c mice. Tumor volumes were determined and subsequently extracted after mice sacrifice. The immunohistochemistry staining was performed for analysis of Ki-67 expression (a tumor proliferation marker) in tumor masses. The expression of IL-12 and IFN-γ were studied using real-time polymerase chain reaction and immunoblotting.

Results: The group treated with IL-12 loaded chitosan nanoparticles indicated decreasing of tumor mass volume (P<0.001). The results of western blotting and real-time PCR showed that the IL-12 expression was increased in the group. Immunohistochemistry staining indicated that the Ki-67expression was reduced in the group treated with IL-12 loaded chitosan nanoparticles.

Conclusion: IL-12 gene therapy using chitosan nanoparticles has therapeutic effects on the regression of tumor masses in fibrosarcoma mouse model.

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Introduction

Fibrosarcoma is a soft tissue sarcoma derived from fibrous connective tissue (1). The immunological approaches for cancer treatment are specific for tumor cells and do not damage the normal cells (2, 3). Cancer immunotherapy consists of the different treatment approaches and incorporates the great specificity of the adaptive immune system (T cells and antibodies) and the various and strong cytotoxic weaponry of both acquired and innate immunity. The cancer immune-therapy boosts the weak host immune response against developing tumors (4-6). Cytokines are immune-modulator agents that are secreted by specific cells of immune system and other cells of the body. The multiple actions of cytokines include several effects on the immune system cells such as the modulating the inflammatory reactions (7). Cancer gene immune-therapy is a delivery of cytokine genes to tumor cells to modify the local tumor environment to induce anti-tumor immune responses (8). In comparison to the therapeutic protein therapy, delivery of cytokine genes avoids the necessity for production and purification of large quantities of recombinant proteins. Moreover, gene immunotherapy is capable of delivering cytokines in a more efficient and safe manner (9). Interleukin-12 (IL-12), is a 74 kDa heterodimeric cytokine, consisting of 35 kDa (p35) and 40 kDa (p40) subunits (10-11). IL-12 was characterized as a natural killer-stimulating factor (NKSF) and cytotoxic lymphocyte maturation factor (CLMF) by Trinchieri's group in 1989 and Gately's group in 1990. This cytokine is produced by macrophages, monocytes, and dendritic cells (DCs) (11-13). Investigations have showed that IL-12 possesses superior antitumor effects compared to the other cytokines. Also, the researches demons-trated that IL-12 can be efficacious in prevention of primary tumor growth (14). IL-12 is a multifunctional cytokine which can develop the multifunctional effects, including the

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rise in the proliferation and cytotoxic activity of T cells and NK cells (12, 15, 16). Moreover, IL-12 possesses potent anti-angiogenic activity produced by neutrophils, macrophages and dendritic cells (17). The endogenous production of IFN-γ is needed for the antitumor effect of IL-12 in most cases. On the whole, IL-12 is one of the potent cytokines for cancer immunotherapy (15, 16).

Generally, the application of recombinant Il-12 in humans has been very limited. Therefore, the local administration of gene encoding this cytokine, may be less toxic compared to the systemic delivery of IL-12 as a recombinant protein (18).

A wide variety of viral and non-viral vectors have been developed to transfer genetic materials to cancerous cells. In fact, nano-sized particles (NPs) offer increased cellular uptake, deeper tissue penetrability and targeting of particular cell types. In addition, some NPs are capable of interacting with and crossing mucosal surfaces, escaping endolysosomal compartments and sustaining release of the gene within the cell (19).

On the whole, there are very limited investigations about the application of polymers as non-viral gene delivery systems for IL-12 gene therapy. Nanoparticle-mediated IL-12 cancer gene therapy has been reviewed thoroughly elsewhere (20). Chitosan is a linear biodegradable, biocompatible and non-allergenic polysaccharide. This polymer is composed of randomly distributed N-acetyl-D-glucosamine and β-(1, 4)-linked D-glucosamine and is able to bind to nucleic acids. The nanoparticles prepared using chitosan showed the ability of entering the nuclear membrane and delivering the therapeutic agent directly into the nucleus (21). Chitosan-DNA complexes are capable of protecting DNA from digestion by DNase I *in vitro*. Moreover, this polymer is able to swirl across the membrane bilayer and improve the cellular uptake of the DNA containing complexes (22).

Hence, considering the privileges of chitosan, we aimed to investigate the effects of gene therapy via cells transfected with mIL-12 loaded chitosan nanoparticles in the regression of tumor masses in fibrosarcoma mouse model.

Materials and Methods

Plasmid amplification and isolation

The IL-12 expression vector, pUMVC3-mIL-12, was purchased from Aldevron company (Fargo, ND). The plasmid DNA is 6247 bp in size and contains CMV IE promoter. PUMVC1-IL-12 was amplified in *Escherichia coli DH5α* strain which was obtained from Iranian Biological Research Center (IBRC), Tehran, Iran and then extracted according to TENS protocols. The purified plasmid was detected by agarose gel electrophoresis and the DNA concentration was quantified by the UV spectrophotometer (Shimadzu, Japan) at 260 nm.

Preparation of pUMVC3-mIL-12 loaded chitosan nanoparticles

Chitosan-DNA nanoparticles were prepared using a complex coacervation process (9). Briefly, chitosan was dissolved in 1% acetic acid with gentle heating, and the pH of the solution was adjusted to 5.5–5.7. The solution was diluted to concentrations ranging from 0.01 to 1.0% of chitosan (w/v). The chitosan solution was readjusted to pH=5.5 and sterile-filtered through a 0.45 μm filter. Equal volumes of different chitosan solutions and DNA solutions of 100 -200 μg/ml in 25 mM of sodium sulfate solution at 55 ˚C were rapidly mixed and vortexed at maximum speed for 45 secs. The resulting polyplexes were allowed to stand at room temperature for 30 min for stabilization.

TEM of IL-12 loaded chitosan nanoparticles

Particle morphology of IL-12 loaded chitosan nanoparticles was determined by transmission electron microscopy (TEM). One drop of the DNA/chitosan nanoparticles was put on the copper grid and stained using 1% uranyl acetate solution for 5 sec. The grid was let to dry further for 10 min and then analyzed by the electron microscope.

Cell culture

BALB/c mouse fibrosarcoma cells (WEHI-164) were obtained from the Pasteur Institute (Tehran, Iran). The *WEHI-164* cells were cultured in RPMI-1640 medium (Sigma, Germany) supplemented with 10% fetal bovine serum (Sigma, Germany), in presence of penicillin (100 U/ml), streptomycin (100 μg/ml), (Sigma, Germany), and incubated in humidified incubator with 5% CO_2 at 37 ˚C.

In vitro transfection studies

The cells were trypsinized and seeded into 6-well plate at a density of 4× 10^5 cells/well. The cells were washed with phosphate-buffer solution (PBS) twice prior to the addition of 2 ml RPMI1640 without FBS and antibiotic.

The cells were incubated with chitosan/DNA nanoparticles at the concentration of 5 μg pUMVC3-hIL12/well for 6 hr at 37 ˚C under 5% CO_2 atmosphere. After 6 hr, the nanoparticles were aspirated and replaced with culture medium.

After 48 hr of transfection, supernatants were harvested and IL-12 release was confirmed by ELISA, Mouse IL-12 ELISA kit (Koma Biotech, Korea), according to manufacturer's instructions.

Tumor implantation and treatment

Animal experiments were carried out after confirmation of the IL-12 production by tumor cells transfected with pUMVC3-mIL-12. Female BALB/c mice (6 to 8 weeks old) were obtained from the Pasteur Institute, Tehran, Iran. 48 hr after *WEHI-164*

Table 1. Primers used for quantitative real-time PCR

MouseIL-12p40	Forward: 5-' GAGCACTCCCCATTCCTACT- 3' Reverse: 5'-GCATTGGACTTCGGTAGATG- 3'
Mouse IFN-γ	Forward: 5'TCAGCAACAGCAAGGCGAAAA AG-3' Reverse: 5'-ACCCCGAATCAGCAGCGAC TC-3'
GAPDH	Forward: 5'- CCTCGTCCCGTAGACAAAA-3' Reverse: 5'-AATCTCCACTTTGCCACTG-3'

cells transfected with pUMVC3-mIL-12 loaded chitosan nanoparticles, one million transfected cells were injected subcutaneously into the right flank of the BALB/c mice to establish tumor in the group treated with pUMVC3-mIL-12 loaded chitosan nanoparticles (case group). Also, cells transfected with naked plasmid (as negative controls) as well as Lipofectamine ™ 2000/ pUMVC3-mIL-12 complexes (as positive controls) were used and inoculated as mentioned above.

The palpable tumors were developed after 10 days. Tumor growth was monitored three times a week with calipers after tumor challenge until the experiment was completed. Tumor volumes (mm^3) were calculated by the formula: $1/2\times (length\times width^2)$.

RNA extraction and real time PCR

Following tumor mass extraction, total RNA was extracted by AccuZol™ reagent (Bioneer, Daedeok-gu, Daejeon, Korea) as described by the manufacturer. Complementary DNA (cDNA) was generated from 1 µg of total RNA by using of oligo-dT primer and MMLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's recommendations. qRT - PCR was performed with SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan) in the Rotor-Gene™ 6000 system (Corbett Life Science, Mortlake, NSW, Australia). The PCR was done in a 20 µl reaction system containing 12 µl of SYBR green reagent, 0.2 µM of each specific primer (MouseIL-12p40, Mouse IFN-γ and GAPDH), 1 µl of cDNA template and 6 µl of nuclease-free distilled water. GAPDH was used as an internal expression control. The primer sequences were indicated in Table 1 and obtained from Bioneer. The initial denaturation step at 95 ˚C for 10 min was followed by 45 cycles at 95 ˚C for 20 sec and 60 ˚C for 1 min. Relative IL-12 mRNA expression was calculated with the $2^{-(\Delta\Delta CT)}$, using GAPDH as a reference gene.

Western blotting

The purified anti-mouse IL-12 (1:1000) (biolegend), and anti-β-actin (l: 1000) (Sigma) were used as primary antibodies. Cell lysis and protein preparation of the removed tumors were carried out using RIPA-B buffer (0.5% Non-idetP40, 20 mM Tris, (pH 8.0), 50 mM NaCl, 50 mM NaF, 100 µM Na3VO4, 1 mM dithio-threitol, and 50 µg/ml phenyl-methylsulfonyl fluoride).

The protein concentration was quantified using a UV spectrophotometer. Protein samples were fractionated in the 12% polyacrylamide gel and transferd to the nitrocellulose membrane. The membrane was blocked with 3% skim milk for 1 hr at room temperature and incubated with primary antibody at 4 ˚C overnight. After extensively washing, the membrane was incubated with (Rabbit Polyclonal secondary antibody to Rat IgG (HRP-conjugated)), (abcam). The results were visualized using the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ, USA) and exposed to the autoradiography film (Ko-dak XAR film).

Immunohistochemistry

Immunohistochemical assays were performed to detect Ki67 protein expression. Ki67 is a nuclear protein and is considered as a proliferation biomarker in tumor masses. Four-micrometer frozen sections were cut, air-dried, fixed in acetone, and rehydrated in PBS containing 0.05% Tween-20. Non-specific binding sites were blocked by blocking buffer which is pre-formulated with Tween-20 for 30 min. Slides were incubated with primary antibody (Purified anti-mouse Ki67), (Biolegend), for 60 min. Subsequently, slides were washed in PBS containing 0.05% Tween 20 and then slides were incubated with HRP labeled secondary antibody (Rabbit Polyclonal secondary antibody to Rat IgG (HRP-conjugated)), (abcam), for 30 min. H_2O_2 was added to DAB solution (substrate solution) and DAB and H_2O_2 were added to the slides for 5 min. The slides were consequently washed with PBS containing 0.05% Tween-20 and studied by invert microscopy.

Statistical analysis

One-way ANOVA was used to determine significant differences between groups. Statistical analysis was carried out by GraphPad Prsim software version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. $P<0.05$ was considered to be statistically significant.

Results

Particle Size and Morphological Characterization

The resulting polyplexes were nano-sized (381.83±82.77 nm) and their zeta potential was 14.77±2.35 mV (9). A transmission electron microscopy (TEM) image of IL12 loaded chitosan nanoparticles is shown in Figure 1. The particles were spherical and nano-sized.

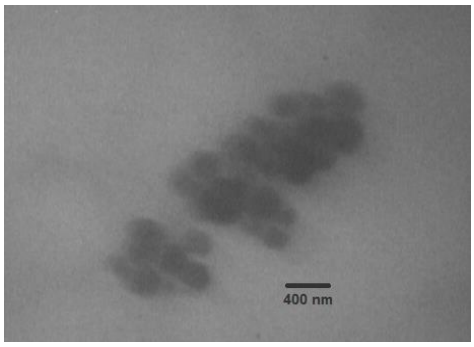

Figure 1. A transmission electron microscopy (TEM) image of Interleukin-12 loaded chitosan nanoparticles

Antitumor effects of IL-12 loaded chitosan nanoparticles in vivo

To test the antitumor response induced by IL-12 secreted in site, BALB/c mice (n=7) were injected subcutaneously with fibrosarcoma/ IL-12 loaded chitosan nanoparticles cells (1×10^6), and monitored for tumor growth three times a week. At the end of observation (21 days after tumor inoculation), the volume of tumor masses of the mice injected with fibrosarcoma/ IL-12 loaded chitosan nanoparticles cells, were less than negative control group (P<0.001). This result indicated the anti-tumor effect of the pUMVC3-mIL-12 loaded chitosan nanoparticles in the regression of tumor masses (Figure 2).

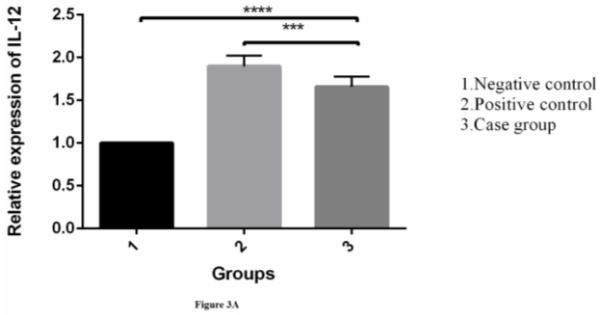

Figure 3A. Relative expression of Interleukin-12(IL-12) gene. IL-12 gene expression in the case group was up-regulated (compared to the negative control group) by a mean factor of 1.7 (P<0.0001);***P<0.001; ****, P<0.0001

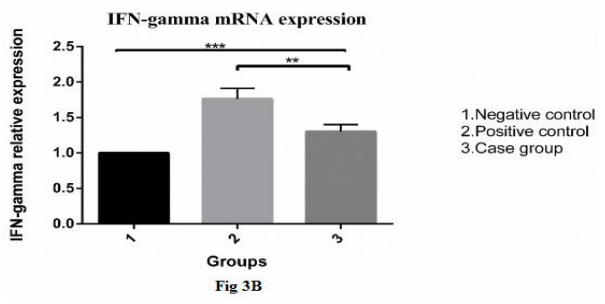

Figure 3B. Relative expression of Interferon gamma (IFN-γ) gene. IFN-γ gene expression in the case group was up-regulated (compared to the negative control group) by a mean factor of 1.3 (P<0.001). ** P<0.01; ***, P<0.001

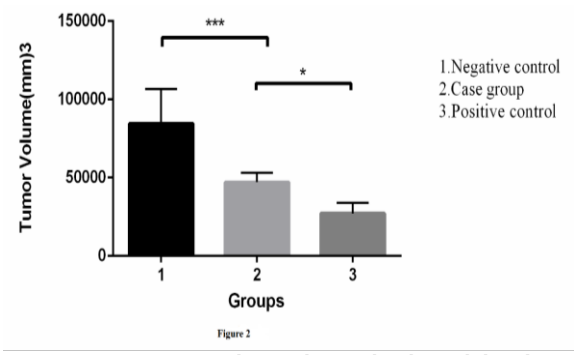

Figure 2. Tumor mass volume. The results showed that the tumor mass volume was significantly reduced in the case group compared with the negative control group (P<0.001); * P<0.05, *** P< 0.001

Expression of IL-12 & IFN-γ mRNA and protein in tumor tissue

To investigate whether fibrosarcoma/ IL-12 loaded chitosan nanoparticles cells can express IL-12 *in vivo*, total RNA was extracted from tumor masses and cell lyses were prepared. The results of real-time PCR indicated that the expression of IL-12 and IFN-γ were enhanced in the case group compared with the negative control group (P<0.0001, P<0.001) (Figure 3). And the results of immunoblotting showed that the expression of IL-12 and IFN-γ were enhanced in the case group compared with the negative control group (Figure 4).

Expression of Ki67 in tumor sections

Ki67 is a nuclear protein which used as a proliferation biomarker. The expression of Ki67 in tumor masses was studied with immunohistochemistry staining. The results showed that the expression of Ki67 in the case group was less than negative control group (P<0.05) (Figure 5).

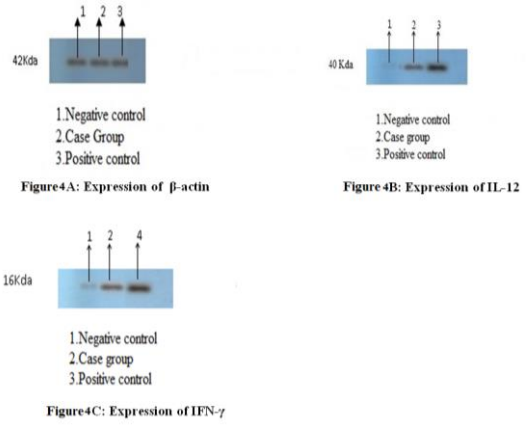

Figure 4. The cytokines expression results in Western blot. IL-12 and INF-γ expression has been proved by Western blotting analysis. A: Proteins were equalized by use of β-actin expression: B: Western blotting results showed that the expression of IL-12 was enhanced in the case group compared with the negative control group. C: Western blotting results showed that the expression of IFN-γ was enhanced in the case group compared with the negative control group

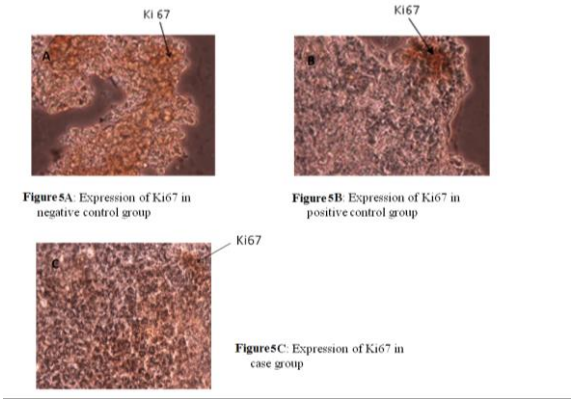

Figure 5. Expression of Ki67 in tumor tissue.
A: Expression of Ki67 in negative control group
B: Expression of Ki67 in positive control group
C: Expression of Ki67 in case group

Discussion

Cancer gene therapy using cytokines genes is a promising approach resulting in potential immune responses against the cancerous cells (23). Immunotherapy-based strategies for cancer treatment try to enhance the local and systemic anti-tumor immune responses. IL-12 protein has been shown to have a superior antitumor activity rather than the other cytokines. This protein results in the induction of an efficient cellular immune response against tumor, which includes a local increase in the inflammatory Th1 cytokines in the tumor microenvironment and a systemic protective immunity, is a promising approach for cancer immunotherapy (24). IL-12 gene delivery has shown prominent antitumor effects (25-30). Cancer cells transduced *in vitro* with IL-12 cDNA, have demonstrated induced antitumor immune responses against MCA207 (murine sarcoma cell line). Besides, active immunization of the established P1.HTR tumor setting by IL-12, showed co-stimulation of sufficient B7 (T cell co-stimulator molecule) of the host, so that not exogenous administration is not required (31, 32).

Remarkable advance in development of non-viral gene carriers has been made to overcome the current issues associated with viral vectors such as safety, immunogenicity and mutagenesis (31). Cationic phospholipids and cationic polymers are two major types considered in gene delivery system. Both have electrostatic interaction with negatively charged DNA and produce lipo/ polyplex complexes (33). The advantages of polyplexes over lipoplexes include low cytotoxicity and more stability (34).

Chitosan is one of the cationic polymers that would be a suitable candidate for gene delivery due to its biocompatibility, biodegradability and cationic potential. Furthermore, chitosan can protect DNA against degradation by nuclease. For chitosan preparation, sonication or organic solvents dose not required, so this led to minimum possible damage to DNA during complexation. Lower molecular weight chitosan causes less cytotoxicity and more solubility in water (35).

Chitosan is a low toxic gene transfer vector and due to the low cytotoxicity of chitosan, it is feasible to use repeated administrations or increase the dose of the DNA/chitosan complexes [9].

Lipofectamine is considered the commercial gold standard transfection reagent but yet not used for *in vivo* application because of its high cytotoxicity and immunogenicity (35). Lipofectamine is used as a basis of comparison for efficiencies of other gene delivery systems (36). Hence, more research is needed to find a gene carrier which is low toxic and possesses more transfection ability relative to the gold standard, Lipofectamine.

Thus, considering the advantages of chitosan, it can be take into consideration as a candidate for gene delivery studies. Also, nanoparticles as non-viral gene delivery systems have been the subject of numerous investigations in the past few decades. In this study, we investigated the effect of chitosan nanoparticles for delivery of IL-12 gene.

The results of our investigation showed that IL-12 secretion in cancerous cells is an efficient method for achieving a high local concentration of IL-12 with low systemic toxicity. The antitumor effect of IL-12 gene therapy was mostly dependent on NK cells and partly on T cells. The anti-neovascularization effect of IL-12 on tumor cells was also dependent on the two cellular components. It has shown that NK cells and T cells inhibit tumor angiogenesis by production of IFN-γ (37). IFN-γ is a potent activator NK cells and enhances the cytolytic activity of the cells. Our study showed that the tumor volume in the case group was significantly reduced compared with the negative control group (P<001). The results of real time PCR and Western blotting showed that the expression of IL-12 and IFN-γ were enhanced significantly in the pUMVC3-mIL-12 loaded chitosan nanoparticles compared with the control group. Also, gene therapy with pUMVC3-mIL-12 loaded chitosan nanoparticles could decrease Ki-67 expression in the fibrosarcoma tumor masses and this finding showed that pUMVC3-mIL-12 loaded chitosan nanoparticles could reduce cancerous cell proliferation. Ki-67 is a protein related to cell proliferation and is present in all other cell cycle phases except G0. Additionally, a significant intra-tumoral level of IFN-γ was also observed as an inducer of two important antitumor chemokines, IP-10 and MIG (37). MIG expression in the microenvironment of tumor induces T cells and NK cells to exert their antitumor cytotoxic activities (29). However, the anti-tumor effect of gene therapy with pUMVC3-mIL-12 loaded chitosan nanoparticles was less effective than the Lipofectamine™ formulation in the studied gene-cell combination.

In vivo study of chitosan nanoparticels for gene therapy have been carried out by other research

groups. For instance, in 2007, Yang *et al* co-administrated chitosan-IL-2 nanoparticles (CNP-IL2) and paratyphoid vaccine intramuscularly to BALB/c mice in order to test the adjuvant effect of CNP-IL2. The results indicated that encapsulation of cytokine genes with chitosan nanoparticles can effectively enhance cytokine expression *in vivo* and increase the cytokine gene bioactivity (38). In another study, Zhang *et al* showed that chitosan-polyaspartic acid-5-fluorouracil could inhibit tumor growth rate in nude mice with gastric cancer more effective than 5-Fu alone (39).

Taken together, chitosan nanoparticels can be considered as a promising candidate for gene delivery.

Conclusion

In this study, we aimed to investigate the effects of gene therapy using mIL-12 loaded chitosan nanoparticles in the regression of tumor masses in fibrosarcoma mouse model. We demonstrated that the local IL-12 gene delivery into tumor tissue resulted in immune response to the tumor cells. Therefore, gene delivery by chitosan/DNA nanoparticles could be a useful non-viral system in cancer gene therapy.

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