RESEARCH ARTICLE

Preparation and Evaluation of anticancer activity D-glucosamine Nanoparticles on Metastatic cancer Model *in vivo*

Neda Soleimani [1]*, Masoumeh Tavakoli Yaraki [2], Baharak Farhangi [3]

[1] Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran

[2] Department of Biochemistry, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

[3] Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

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ABSTRACT

Objective(s): Breast cancer imposes a highest rate of malignancy among the women all around the world. Chitin and its derivatives such as D-glucosamine-carboxymethyl chitin and Di-hydroxy propyl chitin have immune-modulating effects and influence on innate and acquisitive immunity which lead to cell activity enhancement. The aim of this study was investigating the effect of D-glucosamine nanoparticle on immune responses such as the changes in cytokines type 1 and 2 level in tumoral mice.

Methods: Nanoparticles were synthesized by ionic gelation method and characterized by DLS and SEM methods. Tumors were induced in experimental mice and subsequently treated with nanoparticles. Then, the production of cytokine interferon-γ (IFN-γ) and interleukin 4 (IL-4) were evaluated.

Results: The obtained results showed a significant increase in the level of IFN-γ production in the mice group treated with nanoparticles compared to control groups. Additionally, there was a reduction in the level of IL-4 and tumor size in the test group.

Conclusions: D-glucosamine nanoparticles can be proposed as a stimulator of the immune system and a promising compound for cancer treatment in the future.

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INTRODUCTION

The uncontrolled cell proliferation caused by environmental factors and genetic defects can leads to cancer initiation. Various viruses and bacteria are attributed to trigger carcinogenesis as well as other risk factors including chemicals, UV rays, and radiation [1]. Breast cancer imposes the highest rate of malignancy among the women worldwide and is considered as a major cause of death in developed and developing countries [3].

The incidence rate of breast cancer is relatively low in Iran compared to other developing countries; however, due to the recent increased prevalence of breast cancer, it can be considered as the most common malignancy among the women [2]. In spite development of different treatments, surgery is still the first line of therapy for breast cancer. Current therapies of cancer are mostly done with the aim of reducing tumor size; however, their effects fade away after a while, and are not practically effective in survival due to the possibility of recurrence [3,4].

Considerable side effects, low specificity, and the possibility of recurrence are the limitations of these methods. Therefore, there is an increasing demand for alternative treatments being more

* Corresponding Author Email: *n_soleimani@sbu.ac.ir*

efficient and specific with minimum side effects [5,6]. The high prevalence of breast cancer and additional problems caused by disease at young ages, emphasize how critical it is to introduce new therapeutic compounds with immunomodulatory properties and less negative side effects. One of the most-considered compounds in this issue is D-glucosamine [7].

Chitin and its derivatives including D-glucosamine- carboxymethyl chitin and Di-hydroxy propyl chitin have immune-modulating (immunomodulatory) effects and can affect innate and acquisitive immunity which leads to increase cell activity and the secretion of cytokines and chemikones [8,9]. Given the fact that chitin and its derivatives do not exist in the human body, their presence can stimulate the immune system through the of immune cells surface receptors such as Detectin-1, TLR-2, and mannose [10].

D-glucosamine suspensions and particles are able to stimulate the immune system via chemotaxis, macrophages activation, and cytokines secretion. These polymers can increase antibody response and activate cytotoxic T cells and natural killer cells. It has been shown that D-glucosamine has low toxicity in normal cells [10, 11]. This polymer is a biocompatible and biodegradable compound with minimum systemic toxicity [11]. These nanoparticles are effective in directing the immune system to create a specific type of response and enhance the immune response.

In the present study, we aimed to evaluate the immune system responses, especially attenuation of humoral immune as a major factor in anti-tumor defense, toward D-glucosamine nanoparticles in mice model. We have investigated the role of the cell-mediated immunity response cytokines type 1 and 2 (interferon-γ (IFN-γ) and interleukin 4 (IL-4)) in the mice model with metastatic tumor after administrating D-glucosamine nanoparticles as the candidate treatment.

MATERIALS AND METHODS

Materials

Medium-molecular-weight Chi with a degree of deacetylation of about 89% was purchased from Primex (Karmoy, Norway). Sodium nitrite ($NaNO_2$), PF, sodium tripolyphos- phate (TPP), hydrochloric acid, glacial acetic acid, sodium hydroxide (NaOH), sodium metabisulfite, were all purchased from Merck (Darmstadt, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) was purchased from Sigma-Aldrich (St Louis, MO). The mice were obtained from Pasteur Institute (Tehran, Iran). All chemicals were of analytical grade.

Preparation of Nanoparticles

In order to produce nanoparticles, a 1 mg/mL solution of D-glucosamine in acetic acid (1%) was prepared and placed on stirrer (300 rpm) at room temperature. The Tripolyphosphate (TPP) 0.1 % was dissolved in distilled water and added to D-glucosamine solution all at once (flush mix). The volume ratio of D-glucosamine solution to TPP was 1:2. The reaction was continued for one hour in stable condition. Nanoparticle-free D-glucosamine fragments were removed by centrifugation at 18,000 rpm for 30 min at 15 ° C [18, 20].

Characterization of Nanoparticles

Electric charge

Electric charge, homogeneity and distribution of D-glucosamine nanoparticles were measured using ZETA SIZER (Malvern) at pH 6

Scanning electron microscopy (SEM)

The shape and size of nanoparticles was studied using SEM (Iranian Research Organization for Science and Technology) at pH 6. [12, 13, 15].

Animal model studies

Animal studies have been conducted according to relevant national and international guidelines of the Weatherall report, and Institutional Animal Care and Use Committee (IACUC) in Pasteur Institute, Tehran, Iran. 30 female 5-6 weeks old BALB /c mice were obtained from the Pasteur Institute of Iran and were divided into three groups of test and control [14].

Tumor Cell cultured

Mice breast tumor cells (4T1) were obtained from the Pasteur Institute (Tehran, Iran). Cells were grown at 37 °C in an atmosphere of 5% CO_2 and 95% air condition. The 4T1 cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS [17, 18, 19].

Inducing Tumor model and stimulation

The number of 7.10^5 cells were injected to right flank of each mouse. The first group of mice with tumors was treated with D-glucosamine nanoparticles. The second group containing mice with tumors, received normal saline; and the third

group of mice was healthy mice without tumors. The growth rate of tumors was measured daily. For assessing survival rate, the various groups of mice were monitored and investigated during a period of 45-60 days.

Isolation and culture of spleen cells

18 days after treatment with D-glucosamine nanoparticles and saline, the mice were sacrificed and spleen cells were extracted subsequently. Red blood cells were removed using lysis solution. Then a suspension of spleen cells was plated in 24-well culture plates (5×10^5 cells/mL) and cultured in the presence of enriched RPMI medium. The plates were incubated in an incubator containing 5% CO_2at 37°C for 72 hours. Finally, the supernatant was collected and stored at 70 °C. [16].

Cytokine assay

The culture supernatant was collected and the production of cytokines (IFN-γ and IL-4) in spleen cells was evaluated by ELISA method. Measuring range was less than 3 pg/mL and less than 4 pg/mL for IL-4 and IFN-γ, respectively. [17].

Statistical analysis

Statistical analysis was performed using SPSS (windows version 15, SPSS Inc., Chicago, IL, USA). For multiple comparisons, data were analyzed by one-way analysis of variance (ANOVA) and followed by LSD test. The p-value less than 0.05 was considered significant. The results have been expressed by mean ± standard deviation (SD).

RESULTS

Nanoparticle Size

The size, dispersion and homogeneity of D-glucosamine nanoparticles was measured by Dynamic Light Scattering (DLS) (Malvern). The average size of D-glucosamine nanoparticles at pH 6 were determined to be 210 nm (Fig. 1). DLS data of this sample demonstrated that more than 95% of the particles were at 210 nm size range, whereas about 5% of the samples had a size range from 20 to 70 nm.

Electric charge

Electric charge, homogeneity, and distribution of D-glucosamine nanoparticles were investigated using zeta sizer (Malvern). The Fig. 2 shows the

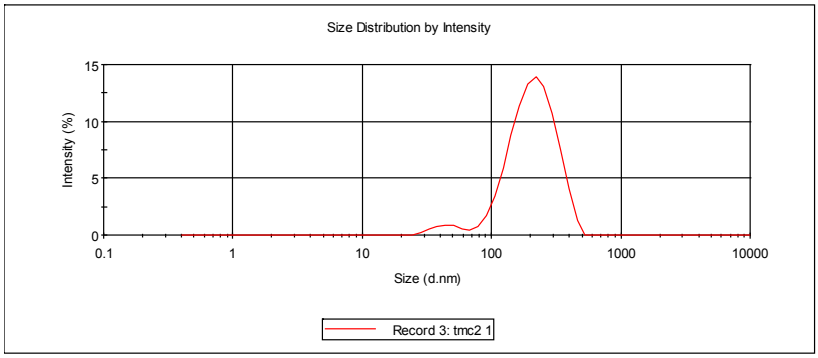

Fig. 1. The size distribution of D-glucosamine nanoparticle measured by Dynamic Light Scattering (DLS)

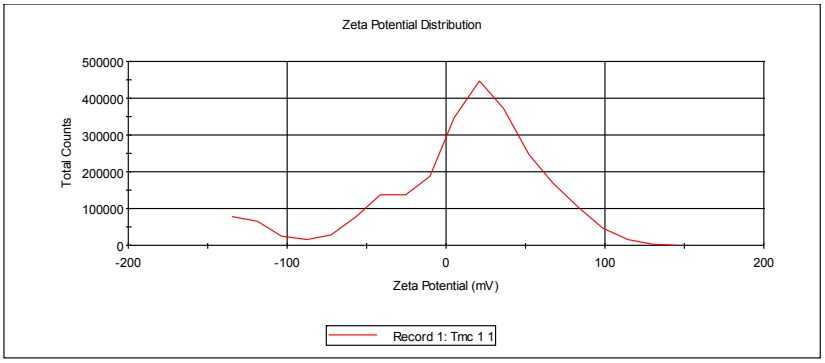

Fig. 2. D-glucosamine nanoparticles electric charge analysis with zeta sizer

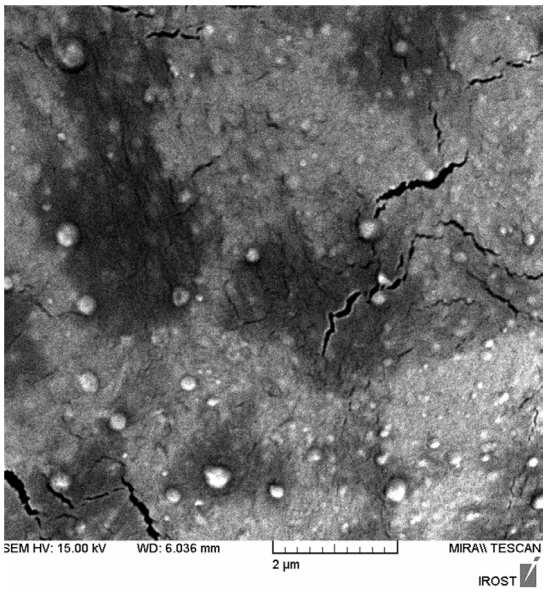

Fig. 3: SEM image of D-glucosamine nanoparticles carrying protein

electric charge of D-glucosamine nanoparticles at pH 6 measured in triplicate by applying a potential of about +11 mV. DLS data indicated a single peak in the curve that showed all particles had homogeneous distribution.

Scanning electron microscope SEM

The result was show that D-glucosamine nanoparticle has spherical shape and the size of nanoparticle was in agreement with the estimated size obtained from DLS analysis in Fig. 3.

Cytokine assay

To evaluate the effect of D-glucosamine nanoparticles on the immune response in the mice model with tumor, the cytokine production level was measured in spleen cell culture supernatant of mice groups. According to the results in Fig. 4, the level of IL-4 in treated mice with nanoparticles was significantly decreased in comparison with control group ($p < 0.05$).

The IFN-γ level of different mice groups is also shown in Fig. 5. According to the results, the mice group treated with nanoparticles has shown significant increase in the levels of IFN-γ compared to untreated mice and normal group of mice ($p<0.05$).

Tumor size and survival study

In this study, before and after each treatment, the size of tumors was measured with digital calipers device and their volume were also determined., The results obtained 12 days after final treatment with D-glucosamine Nanoparticle (in the end of five doses) showed that these nanoparticles significantly inhibited the tumor growth in comparison with the control group ($P<0.05$). To evaluate the tumor treatment efficacy of this compound, the tumor volume was measured by a digital caliper device day by day. As shown in Fig. 6, a time related increase in tumor volume was observed in the control untreated group until day 56.

DISCUSSION

The data obtained from chemical properties of D-glucosamine have revealed that this polymer can be used for drug delivery, especially for releasing macromolecules [6,7]. From a technical point of view, chitosan's properties such as solubility in water and positive surface charge are very valuable in biomedical applications [12]. These features enable D-glucosamine polymer to contact with negatively charged macromolecules or charged cell membrane in aqueous environments. The

Fig. 4: The production of IL-4 levels in different mice groups under study (mean±SD) ($P> 0.05$)

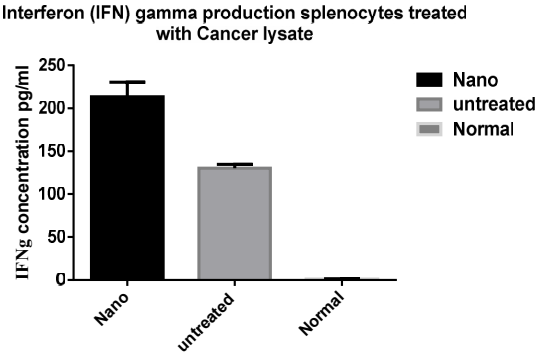

Fig. 5: The production of IFN- γ levels in different mice groups under study (mean±SD) ($P> 0.05$)

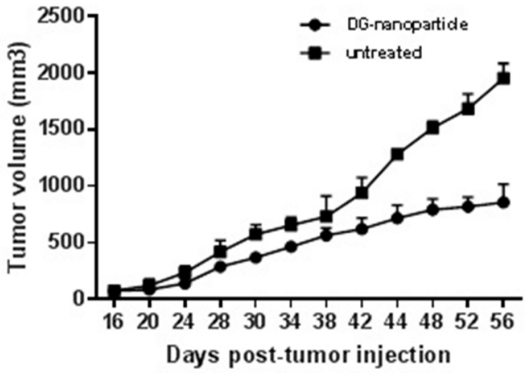

Fig. 6. 4T1 Xenograft tumor cells growth (average volume of the tumor (mm3) ± standard error)

use of polymers such as D-glucosamine for drug delivery to the appropriate location is substantial for biological systems. The application of D-glucosamine nanoparticles in some study was shown that as a drug carrier [20]. D-glucosamine nanoparticles were used as protein carrier for tetanus vaccine by Vila *et al.* [21]. Hosseinzadeh *et al.* used D-glucosamine nanoparticles as a drug delivery system for cancer treatment [22].

In this study, we have examined the effects of D-glucosamine nanoparticles on breast cancer in mouse and its possible effect on the immune system response *in vivo*. Nanoparticles in the size range of 100-300 nm can stimulate the immune system. D-glucosamine nanoparticles were determined with a size of about 210 nm. The SEM analysis revealed the same particle size range as that obtained by DLS.

As acid TPP solutions contains both H+ and multivalent tripolyphosphate ions, free amine group on chitosan molecule will be protonated when chitosan contacts acid TPP solution. Then the protonated amine groups on different or same chitosan molecules can be cross-linked by negative charged multivalent TPP ions and was created self-assemble nanoparticle.

D-glucosamine nanoparticles can stimulate the immune system through chemotaxis, macrophages activation, and phagocytosis. The nanoparticles with the size mentioned above can induce the production and secretion of cytokines such as IL-4 and IFN- γ. The level of IL-4 in treated mice with nanoparticles was significantly decreased ($p<0.05$) in comparison with mice of control group. This cytokine plays an important role in humoral immune system, as the diminution in production/ secretion of this cytokine shows that the immune system shifts to the immune response type 1. The IFN-γ is a representative of secretory cytokines in cell-mediated immunity; and its production has indicated the stimulation of this system [23]. The mice with tumor which were treated with D-glucosamine nanoparticle showed a significant reduction in the size of tumor compared to other mice groups. This phenomenon can be explained due to the secretion of IFN-γ which was triggered by D-glucosamine nanoparticle [24]. The protective role of IFN-γ has been reported in many types of tumors, therefore IFN–γ has been proven to have anti-proliferative and pro-apoptotic effects on a large number of tumor cells containing its receptor [25]. These effects depend on the activation of STAT1 (signal transducers and activators of transcription) pathway. In addition, IFN-γ can prevent tumor development by inhibiting angiogenesis and inducing the production of some chemokines such as IP 10 (interferon inducible protein 10) and Mig (monokine induced by IFN-γ), which play a key role in limiting the angiogenesis process [26, 27]. It has been well-evidenced that a direct connection exists between anti-angiogenic properties and adjustments of immune responses type 1.

CONCLUSION

Our results have shown that D-glucosamine nanoparticles can as well as increase IFNγ level and decrease of IL4 level. It is cause of reduction in tumor size. Represent the anti-angiogenic properties that is the first evidence in this regards. The unique features of D-glucosamine nanoparticles make them remarkable candidates for various types of drug delivery as well as promising candidates for cancer therapy alongside with other therapeutic options.

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