

The Immunostimulatory Effect of Biogenic Selenium Nanoparticles on the 4T1 Breast Cancer Model: an In Vivo Study

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Abstract Selenium salts as well as elemental selenium nanoparticles are attracting the attention of researchers due to their excellent biological properties. The aim of the present work was to study immunomodulation by applying elemental Se NPs to stimulate the immune response of mice bearing 4 T1 breast cancer tumors. Six- to 8-week-old female inbred BALB/c mice were divided into two groups of test and control, each containing 15 mice. Every day, for 2 weeks prior to tumor induction, selenium nanoparticles were orally administered to the mice at a dose of 100 µg/day. Then, 1×10^6 cells from a 4 T1 cell line were injected subcutaneously to each mouse. Oral nanoparticle administration was continued daily for 3 weeks after tumor induction. Different immunological parameters were then evaluated including cytokine level, delayed type hypersensitivity (DTH) response as well as tumor growth and the survival rates in all treated or nontreated animals. The production of Th1 cytokines, such as IFN- γ and IL-12, in spleen cell culture was increased in the test mice-administered selenium nanoparticles. The DTH response of test mice also showed a significant increase when compared to the control mice. The survival rate was notably higher for the selenium nanoparticle-treated mice compared to the control mice. Our results suggest that selenium nanoparticle administration

can result in considerable induction of the Th1 platform of immune response through the elevation of IFN- γ and IL-12 and may be a cause for better prognosis in mice with tumors.

Keywords Selenium nanoparticles · Th₁ cytokines · 4 T1 cell line · Breast cancer

Introduction

Selenium (Se) is an important micronutrient ion that has broad effects on biological systems, including antioxidant and anticarcinogenic effects, cancer prevention, and antiviral activities [1–4]. The relation between the incidences of different cancers with dietary Se deficiency has been well established in animal models and in human interventional trials [5, 6]. Se ions was shown to exhibit inhibitory effects in various tumor cell lines as well as against chemically induced and transplanted tumors [7, 8]. Also, excellent research works were performed on the preventive antitumor effects of inorganic or organic salts of Se ions in female mice infected by murine mammary tumor virus [9, 10]. Schrauzer et al. showed that both inorganic Se, such as sodium selenite, as well as organic nutritional forms such as L(+)-selenomethionine are effective in reducing breast cancer incidence [5, 9, 10].

The biological effects of Se mostly depend on the incorporation of this metalloid into selenoproteins in the form of the amino acid selenocysteine [1–3]. Many of these selenoproteins are apparently expressed in most, if not all, tissues and living cells including the immune cells that are involved in innate and adaptive immune responses [11, 12]. Deficiency in Se appears to result in

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immunosuppression, whereas supplementation with low doses of Se appears to result in augmentation or restoration of immunologic functions [13, 14].

Elemental selenium is known to exist in various allotropic forms, such as a red amorphous form, a black vitreous form, three red crystalline monoclinic forms (α , β , and γ), and a grey/black crystalline hexagonal (also referred to as trigonal) form, which is also the most stable form, and more allotropes are still being discovered [15–17]. Elemental selenium in the Se^0 state is an insoluble metalloid compound that can be chemically or biologically produced at nanoscale [18]. The toxicity reported for elemental selenium (Se^0) at nanosize is lower than the toxicity of selenate (Se^{+2}) or selenite (Se^{+4}) ions; therefore, selenium nanoparticles (Se NPs) may be good candidates to replace other forms of selenium in nutritional supplements or pharmaceutical dosage forms [19]. Considering this fact, Se NPs have gained some attention in recent years due to their excellent biological properties, which are similar to selenium ion at even lower doses and which show lower toxicity [20]. A new biomaterial consisting of a titanium surface decorated with Se NPs has been shown to provide nanostructured surface features for potentially enhancing bone formation and prevention of cancer recurrence when used in implants for replacement of cancerous bone [21].

As mentioned above, selenium ions have been used as dietary supplements, but because of the high biological activity and lower toxicity of Se NPs, research is now being directed towards Se NPs. Although some studies have demonstrated detrimental adverse effects of selenium ion on human health [1–3], Se NPs still have much potential considering their effects on the different human disorders such as malignant diseases. At present, no immunomodulatory effects of Se NPs have been reported. In the present work, biogenic Se NPs were prepared by a friendly intestinal bacterium (*Lactobacillus plantarum*), extracted and used to stimulate the immune response of mice bearing 4 T1 breast cancer cells.

Materials and Methods

Animals

Six- to 8-week-old female inbred BALB/c mice, each weighing 25–30 g, were obtained from the Pasteur Institute of Iran (Tehran, Iran). They were divided into two groups (test and control), each containing 15 mice. The mice were kept in plastic cages, allowed free access to water, and maintained on a 12:12 h light and dark cycle during the study period. Temperature and humidity were controlled at $23\pm 1^\circ\text{C}$ and $55\pm 10\%$, respectively, and all mice were fed a standard mouse pellet diet. The control mice in this study

were kept separated from the test group but at the same temperature and humidity and were fed the same food.

Preparation of Biogenic Selenium Nanoparticles

The *L. plantarum* strain (ATCC 8014) was purchased from the Persian type culture collection (Iranian Research Organization for Science and Technology, Tehran, Iran). It was inoculated in 10 ml of DeMan–Rogosa–Sharpe (MRS) broth (Merck, Germany) and cultivated overnight (16 h) at 37°C . After this time, selenium oxide solution was added to the culture broth at a final concentration of 200 mg/L and incubation at 37°C was continued for 72 h. Finally, the bacterial cells containing the Se NPs were removed from the culture medium by centrifugation at $4,000\times g$ for 10 min. The resulting pellets were washed with 0.9% NaCl solution by centrifugation and transferred to a mortar. The pellets were frozen by addition of liquid nitrogen and then disrupted with a pestle. The resulting slurry was ultrasonicated at 100 W for 5 min and washed three times by sequential centrifugation ($10,000\times g$, 5 min) using 1.5 M Tris/HCl buffer (pH 8.3) containing 1% SDS and deionized water. The pellets were suspended in deionized water, and the resulting suspension containing Se NPs and cell debris was collected. Approximately 4 ml of the suspension was transferred to test tubes and 2 ml of *n*-octyl alcohol was added to each tube. The mixtures were then shaken vigorously. The two mixed phases were separated by centrifugation at $2,000\times g$ for 5 min and were stored at 4°C for 24 h. The generated Se NPs settled at the bottom of the tubes, and the cell debris remained between the two phases. The lower and upper phases were discarded, and the settled NPs were washed with chloroform, ethyl alcohol, and distilled water [22]. For further characterizations and biological experiments, the cleaned NPs were then resuspended in deionized water and stored at 4°C . The sizes of the obtained Se NP nanoparticles were less than 250 nm, as determined by transmission electron microscopy.

In Vivo Challenge Test

Administration Procedure

Every day for 2 weeks before tumor induction, the Se NPs were orally administered to the each mouse at a dose of 100 $\mu\text{g}/\text{day}$. Following tumor induction, administration was continued daily at the same dose for 3 weeks.

Tumor Induction

The 4 T₁ cell line was used for induction of tumors in inbred Balb/c mice. For this purpose, 200 μl of RPMI-containing 4 T₁ cells at concentration of 1×10^6 cells/ml was injected

into the mammary glands of female mice. All mice were followed until a tumor nodule was observed.

Measurement of Tumor Growth

Tumor growth was measured twice a week by caliper measurement of the tumor length. The volume of each tumor was determined using the formula: $V=0.5 \times d^2 \times D$ [23] where V is the tumor volume (cubic centimeter), d is the shorter diameter, and D is the longer diameter.

Preparing the Tumor Antigen

The tumor antigen was prepared from the tumor of one of the mice. The tumor was removed from the body, dissected into small sections (1 mm³), and homogenized in sterile phosphate buffered saline (PBS). This homogenized sample was washed with sterile PBS and sonicated. The sample was then dialyzed against 1,000 ml of PBS buffer for 24 h at 4°C. After dialysis, the concentrated sample was collected, and the protein concentration was determined using the conventional Bradford method. The sample was applied to stimulate the splenocytes in cytokine production tests and also in a delayed type hypersensitivity (DTH) response assay.

Evaluation of the DTH Response

The DTH response was measured according to a method described by Jin et al. [24]. Briefly, 3 weeks after tumor cell injection, seven mice from each group were challenged with the tumor antigen in the left footpad and with the PBS in the right footpad. Footpad induration was measured at 72 h later using caliper measurements.

Cytokine Determination in Spleen Cell Cultures

Three weeks after tumor cell injection, spleens of eight mice from each group were removed aseptically and dissected. Spleen cells were then collected, and RBCs in the collection were disrupted with conventional RBC lysis buffer. The spleen cell counts were adjusted to 2.5×10^6 cells/ml in RPMI1640 (Gibco Life Technologies, Germany) supplemented with 10% fetal bovine serum (Invitrogen, Paisley Germany), 100 µg/ml streptomycin, and 100 IU/ml penicillin (Sigma, Germany). These cells were then stimulated with 20 µg/ml of the tumor antigen (this amount was determined in a previous study) for 72 h at 37°C in a humidified atmosphere of 5% CO₂. IL-12 and IFN-γ from the spleen cell cultured supernatants were detected using ELISA kits (R&D System) according to the manufacturer's instructions.

Survival Rate

At the end of the study period (3 weeks after tumor cell injection), seven mice from each group (these were used for the DTH assay as well) were kept in standard conditions and fed with standard diet, had free access to water, and maintained on a 12:12 h light–dark cycle until they died. Daily deaths were recorded, and after the last death in both groups, data were analyzed with a Kaplan–Meier test.

Statistical Analysis

All of the statistical analyses except for survival rate were conducted using SPSS 15.0 and Student ANOVA. The survival rate data were analyzed by the Kaplan–Meier test. The values are presented as mean±SD.

Ethical Approval

The experimental procedures carried out in this study were in compliance with the guidelines of the Tehran University of Medical Science (Tehran, Iran) for the care and use of laboratory animals.

Results

Measurement of Tumor Growth

To evaluate tumor growth, the tumors from 15 mice from each group were measured twice a week with calipers and the tumor volume calculated as described in “Materials and Methods”. Data analysis showed a significant ($P=0.05$) decrease in the growth rate of tumor in the test mice when compared to the control mice. This effect could be a result of promotion of Th1 immune responses and enhancement of cellular immunity triggered by Se NPs administration (Table 1).

Evaluation of Delayed Type Hypersensitivity Response (DTH)

The antigen-specific Th1 recalling response was assessed by evaluating the DTH reaction in the tumor antigen rechallenged mice. These mice were challenged with tumor antigen in the left footpad and with PBS in the right footpad 3 weeks after tumor injection. The results showed a greater swelling and thickness in the left footpad 72 h after the tumor antigen challenge in the test group compared with the control group (Table 1).

Cytokine Determination in Spleen Cell Culture

The levels of IFN-γ, IL-12, IL-2, and TNF-α in the spleen cell culture supernatants were measured using a sandwich ELISA

Table 1 Physical examination results: tumor volumes and foot induration in tumor-bearing mice

Groups	Relative tumor volume ^a		Swelling in DTH assay (%) ^b	
	Mean	SD	Mean	SD
Control	2.277*	0.386	9.02	3.36
Se NPs	0.748	0.142	17.46	1.83

^a Related tumor volume: tumor growth was measured twice a week and evaluated by caliper measurement of the tumor length. The tumor volume was calculated with the formula presented in the text. Data represent the means±standard deviations for 15 animals per group

^b DTH responses of mice 72 h after tumor antigen rechallenge. The mice were challenged with the tumor antigen on the left footpad and with the PBS on the right footpad. The results showed a difference in the left footpad thickness and swelling between the test and control mice. Data represent the means±standard deviations for seven animals per group

$P=0.05$ (statistical significance)

assay (R&D System). As Fig. 1 shows, the level of IL-12 was significantly higher in the test group than in the control group ($P=0.001$). Figure 2 shows a significantly increased level of IFN- γ in the test group compared to the control ($P=0.001$). The IFN- γ /IL-4 ratios for test and control groups shown in Fig. 3 indicate that the Th1 type of immune response was more likely involved in the test mice than in the control mice. Figure 4 shows that the level of TNF- α in the spleen cell culture supernatants was elevated significantly ($P=0.05$) in the test group. The IL-2 level was also significantly higher in the test group than in the control group, as shown in Fig. 5.

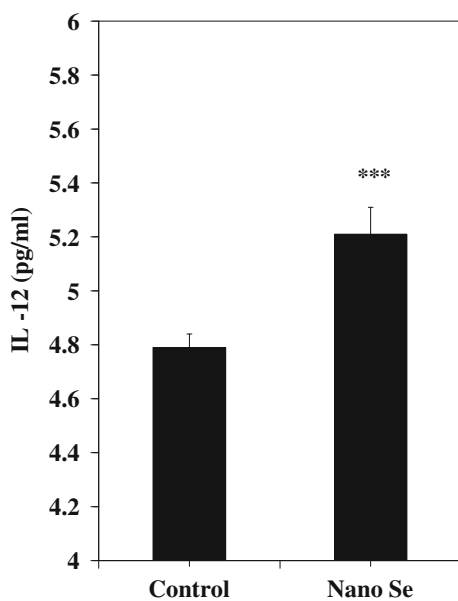


Fig. 1 IL-12 induction by Se NP administration in spleen cell cultures. The spleen cells were cultured for 72 h with tumor antigen stimulation, and the levels of IL-12 in the supernatants were determined by ELISA. Data represent the means±standard deviations for triplicate culture of eight animals per group. Three asterisks indicate $P=0.001$

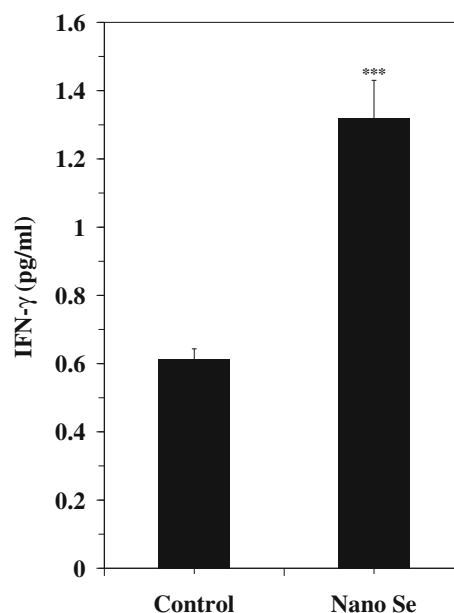


Fig. 2 IFN- γ induction by Se NPs administration in spleen cell cultures. After 72 h of culturing the spleen cells with tumor antigen stimulation, the levels of IFN- γ in the supernatants were determined by ELISA. Data represent the means ± standard deviations for triplicate culture of eight animals per group. Three asterisk indicate $P=0.001$

Survival Rate

Figure 6 shows the results of survival analysis. The test group showed a remarkable decrease in the rate of death when compared to the control group, which indicated that Se NP administration could enhance the survival rate of tumor-bearing mice over a 30-day period.

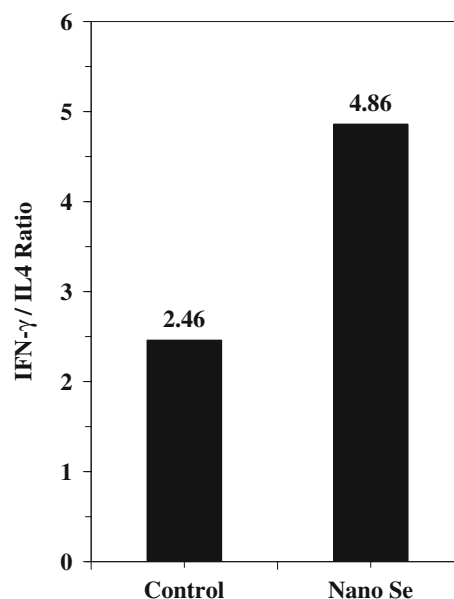


Fig. 3 IFN- γ /IL-4 ratio was calculated by the division of the IFN- γ level of each mouse by its IL-4 level

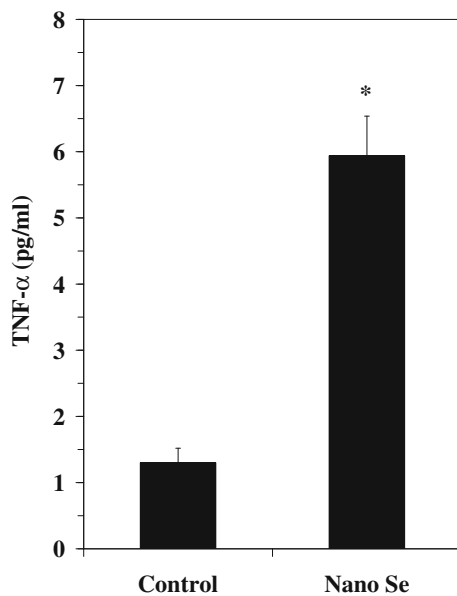


Fig. 4 TNF- α induction by Se NP administration in spleen cell cultures. The levels of TNF- α in the supernatants were determined by ELISA. Data represent the means \pm standard deviations for triplicate culture of eight animals per group. Asterisk indicates $P=0.05$

Discussion

Breast cancer is one of the main causes of death among women worldwide [25]. Breast cancer, like many other types of malignant tumors, is typically treated with invasive methods such as chemotherapy, radiation therapy, and/or surgery, even though most chemotherapeutic regimens

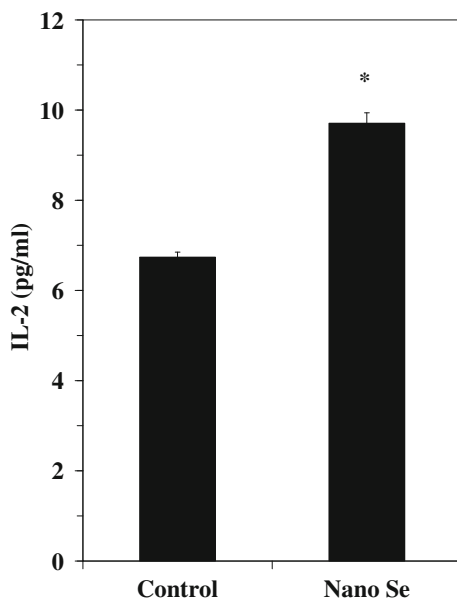


Fig. 5 IL-2 level in spleen cell cultures of mice after 72 h of stimulation with tumor antigen. IL-2 levels were determined using ELISA. Data represent the means \pm standard deviations for triplicate culture of eight animals per group. Asterisk indicates $P=0.05$

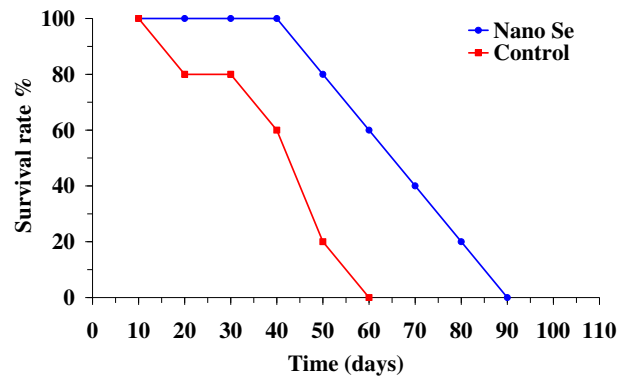


Fig. 6 Effect of Se NP administration on survival rate of tumor-bearing mice. At the end of the study period, seven mice from each experimental group were kept under standard conditions until they died. The rate of death was registered every day, and the obtained data were analyzed with Kaplan–Meier test after the last death in both test and control groups

cause considerable side effects. Depletion of immune cells is one of these side effects and can worsen the patient's situation [26]. Therefore, in the present work, we aimed to study the effect of Se NP administration on the immune responses of mice-bearing 4 T1 breast cancer.

Selenium (“Se”) ions (“selenite”; “selenate”) have been found to affect the development and expression of nonspecific, humoral, and cell-mediated immune responses [13]. Deficiency of these ions results in immunosuppression, whereas supplementation with low doses of selenium ions results in an efficient immune response.

Insoluble Se⁰ (i.e., red elemental selenium) can be prepared at a nanoscale level by reducing selenium ions with higher oxidation states. Red elemental Se NPs not only possess efficiency for upregulation of selenoenzymes, but based on several in vivo and in vitro studies, they also exhibit lower toxicity compared to other selenium compounds [19]. We previously demonstrated that Se NPs can affect iron homeostasis in sheep [27]. In the present research, results of Se NP oral administration showed that the production of proinflammatory cytokines such as IFN- γ and TNF- α increased in tumor-bearing mice, and the level of IL-12 production as a Th1 modulator agent was also raised in comparison to the control mice. IFN- γ is another important Th1 cytokine that has a key role in the activation of natural killer cells (NK cells), which are the first line of immune response against the tumor cells and viral infections. A high level of IFN- γ can also inhibit intratumoral angiogenesis, which has a critical role in tumor development [28]. The IL-12 produced by antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages (MQ), has a well-known central role in polarizing immune responses that skew Th1 balance and also enhance cellular immunity [29]. IL-12 also contributes to the stimulation of both NK and Th1 cells to produce IFN- γ [30]. Increases in the

level of IL-2 in the Se NP-treated mice in the present work also indicate an immunostimulatory effect of this agent.

IL-2 is necessary for the growth and function of T cells and is normally produced by the body during an immune response [31]. The IL-2/IL-2 receptor interaction then stimulates the growth, differentiation, and survival of antigen-selected cytotoxic T cells via the activation of the expression of specific genes [32]. As such, IL-2 is necessary for the development of T cell immunologic memory, which depends upon the expansion of the number and function of antigen-selected T cell clones. IL-2 has been tested in many clinical trials as an immunotherapy for the treatment of cancers, chronic viral infections, and as adjuvants for vaccines [33].

In addition to the increase in the level of these cytokines, an increase in the DTH response was also observed in the Se NP-treated mice. The DTH reaction can also be a sign of Th1 immune responses and can take 2 to 3 days to develop after antigen rechallenge. CD8⁺ cytotoxic T cells and CD4⁺ helper T cells recognize antigen in a complex with either type 1 or 2 major histocompatibility complex (MHC). The APCs in this case are MQs that secrete IL-12, which stimulates the proliferation of further CD4⁺ T cells. The CD4⁺ T cells secrete IL-2 and gamma-interferon, further inducing the release of other type 1 cytokines and thereby mediating the immune response [34].

Conclusion

A more important result seen in the present study is the decrease in the tumor-related volume following Se NP administration, which could be related to the development of an efficient antitumor immune response with a Th1 bias. Lastly, the increase in the survival rate of mice treated with Se NPs, which resulted in a better immune function against breast tumors, can indicate that consumption of nano-red elemental selenium might have some beneficial effects for patients suffering from tumor development. In conclusion, the results of the present research suggest that preventive Se NP oral consumption could promote a more effective immune response against tumors. However, more studies remain to be conducted to investigate other potential mechanisms of action.

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