Oral administration of synthetic selenium nanoparticles induced robust Th1 cytokine pattern after HBs antigen vaccination in mouse model

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**KEYWORDS**
Selenium nanoparticles; Hepatitis B vaccine; IFN-\gamma; Th1 immune response

**Summary**
Hepatitis B virus (HBV) infection is known as a life-threatening liver infection and leads to chronic liver disease if left untreated. Nevertheless, the prevalence of HBV infection has been reduced by an approved vaccination approach using recombinant Hepatitis B surface Antigen (HBsAg) and Alum, known as the HBV vaccine. Alum can be used as an adjuvant to increase HBsAg immunogenicity as a strong Th2 stimulator. There is a vital need to stimulate Th1 immunity by HBsAg

**Abbreviations:** HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; SeNPs, selenium nanoparticles; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; HEPES, 4-2-hydroxyethylpiperazine-1-2-ethanesulfonic acid; FBS, fetal bovine serum; BrdU, 5-bromo-2′-deoxyuridine; PBS-BSA, phosphate buffered saline with bovine serum albumin; PBMCs, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; NK, natural killer; TMB, tetra-methyl benzidine; SI, stimulation index; HRP, horseradish peroxidase.

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vaccination; however, the present vaccine does not induce a prophylactic immune response in some groups. The main aim of the present study was to induce a Th1 cytokine pattern and stimulate an immune response after HBsAg vaccination. Experimental mice were fed selenium nanoparticles (SeNPs) and were later immunized with 5 μg of Hepatitis B Vaccine. After a period of 30 days, the experimental animals were given two booster doses of SeNPs during their vaccination course. Group one, i.e., the control vaccine group, was only administered the HBsAg vaccine. The two treated groups, Groups 2 and 3, were daily fed different doses of SeNPs (100 μg and 200 μg, respectively) via gavage. Group four was considered the control group and was only given phosphate buffered saline (PBS). Lymphocyte proliferation, IFN-γ and IL-4 levels, total antibody and the isotypes of IgG1, IgG2a, IgG2b, and IgM were measured by Enzyme Linked Immunosorbent Assay (ELISA). The administration of SeNPs and the HBs antigen vaccine affected the lymphocyte proliferation; moreover, the total antibody responses also increased the IFN-γ level and induced a Th1 response.

Conclusions: The present study proposed that the administration of SeNPs with a conventional HBs antigen vaccine induces a better immune response with a Th1 bias. © 2016 King Saud Bin Abdulaziz University for Health Sciences. Published by Elsevier Limited. All rights reserved.

Background
Hepatitis B virus (HBV) infection is a hazardous liver infection. Globally, over 350 million people suffer from persistent HBV infection. At least 600,000 patients die of hepatitis B virus (HBV)-related diseases, including Chronic Hepatitis B (CHB), Liver Cirrhosis (LC), and Hepatocellular Carcinoma (HCC) annually [1]. A significant reduction in HBV infection prevalence has been achieved by an approved vaccination approach using recombinant Hepatitis B surface Antigen (HBsAg) [2]. However, some diabetic and hemodialysis patients and 12–21% of healthy vaccinated adults do not develop an adequate response following the HBsAg vaccine [3]. It has been demonstrated that a Th1 immune response is related to the successful development of humoral immune responses against HBs antigen [4]. One important immunologic finding in the non-responder patients is a defect in IFN-γ production due to the interference of the virus with cytokine production, which finally weakens the cellular immune response and results in disease progression [5], stimulation of cellular immune response and the Th1 cytokine platform that is critical for the disease control and treatment. This strategy, known as therapeutic vaccination, is effective in patients and viably controls the disease. Although alum is used as an ordinary adjuvant in the HBs vaccine formulation for its induction of a strong humoral immune response, it failed to induce cellular immune responses [6]. Herein, the utilization of an immunomodulator agent in parallel with HBs vaccination may be useful to induce both cellular as well as humoral immune responses. Selenium plays a dominant role in many processes such as immune system modulation, antioxidant and anticarcinogenic effects, cancer prevention, and antiviral activities [7].

Elemental selenium in the Se⁰ state is an insoluble metalloid compound chemically or biologically produced at the nanoscale. Selenium nanoparticles (SeNPs) have gained attention in recent years due to their excellent biological properties, which are similar to selenium ions but in even lower doses and with less toxicity [8]. According to previous studies, the administration of SeNPs to mice increased Th1 immune responses and triggered cytokine production such as IFN-γ, TNF-α, IL-12 and IL-2 [9]. This immunologic profile clearly showed that selenium possesses the ability to polarize the immune system toward a Th1 pattern and thereby increase the efficacy of vaccines against many viral and bacterial pathogens specifically controlled by cellular immune responses. This in turn authenticated the relationship between IFN-γ release and successful antibody secretion [4].

Therefore, in the present study, we hypothesized that the oral feeding of SeNPs to mice immunized with HBsAg will result in IFN-γ secretion in addition to improving their humoral immune responses.
Materials and methods

Selenium nanoparticles’ preparation and characterization

A 5.2 mM selenium dioxide solution (Merck, Germany) was prepared and an aqueous ascorbic acid solution (5.2 mM) was slowly added to the mixture with continuous stirring at 300 rpm using a laboratory magnetic stirrer. Later, the mixture was centrifuged and washed three times with double-distilled water. The NPs’ surface and elemental composition were studied by field-emission scanning electron microscopy (FESEM) equipped with energy-dispersive X-ray spectroscopy (EDS). The NPs were coated with gold and mounted on specimen stubs. MIRA 3 FISEM (MIRA 3 TESCAN, USA) was operated at 15 kV to analyze the samples. Moreover, the EDX was applied by focusing on a cluster of NPs. The surface charge of the nanoparticles was determined by Malvern Zetasizer (Malvern Instruments GmbH, Herrenberg). A stock solution of SeNPs was prepared (1 mg/ml) and used for further oral administration in 100 and 200 μl doses.

HBV vaccine and HBs Ag

Conventional HBV vaccine (HBs-Ag formulated in Alum adjuvant) and HBs-Ag supplied by the Pasteur Institute of Iran (Karaş, Iran) were stored at 4°C until use.

Experimental animals

Sixty six-to-eight-week-old inbred BALB/c mice were purchased from the Pasteur Institute of Iran (Karaş, Iran). The mice were housed for a period of seven days prior to the experiments, given free access to food and water and maintained in standard conditions with a 12 h/12 h light/dark and at 18–20°C. All experiments were in accordance with the Animal Care and Use Protocol of Tehran University, Iran.

Immunization and study planning

The mice were divided into four groups with each group containing fifteen mice, and the oral feeding was carried out with synthetic SeNPs for a period of 60 days. After 30 days of administration, the mice were immunized subcutaneously with 5 μg of HBs vaccine in a total volume of 250 μl. After a period of 14 and 28 days, mice were re-administered similar concentrations of booster doses. The mice in group one, which was considered the vaccine control group, received only the Hepatitis B vaccine in the mentioned course. In group 2, the mice were fed 100 μg of synthetic SeNPs for 30 days before and 30 days after immunization. In group 3, the mice were fed 200 μg of synthetic SeNPs for 30 days before and 30 days after immunization. In group 4, the mice were considered the PBS control group and were fed only phosphate buffered saline (PBS).

Lymphocyte proliferation

Two weeks after the final immunization, the cell suspension was prepared by dissecting the spleen in cold PBS containing 2% Fetal Bovine Serum (FBS) under aseptic conditions. Red blood cells were lysed by the addition of 5 ml lysis buffer on cell pellets. A single-cell suspension was prepared and adjusted to 3 × 10^6 cells/ml in RPMI-1640 (Gibco, Germany). Next, the suspension was supplemented with 10% FBS, 4 mM L-glutamine, 25 mM 4-2-Hydroxyethylpiperazine-1-2-Ethanesulfonic Acid (HEPES), 0.1 mM non-essential amino acid, 1 mM sodium pyruvate, 50 μM ZME, 100 μg/ml streptomycin and 100 IU/ml penicillin. Afterwards, 100 μl of a suspension of 3 × 10^5 cells was dispensed in 96-well culture plates (Nunc, Denmark) and stimulated with 5 μg/ml of HBs-Ag as recall antigen. Phytohemagglutinin-A (5 μg/ml; Gibco, Darmstadt, Germany) was used as the positive control, and the un-stimulated wells were used as negative controls. All of the experiments were repeated in triplicate. After 72 h of incubation at 37°C in a 5% CO₂ humid incubator, the cells were pulsed with 20 μl of 10X 5-Bromo-2’-deoxyuridine (BrdU) (Roche, Germany) per well, and culture incubation continued for another 24 h. At that time, the plates were centrifuged at 300 × g for 10 min, and the culture medium was removed and dried at 60°C. Later, by the addition of 200 μl of fixation/denaturation buffer, the cells were permeabilized, and 100 μl of anti-BrdU antibody was added to each well. The plates were washed four times with PBS buffer, and Tetra-Methyl Benzidine (TMB) solution was added to each well. The reaction was continued for 10 min and finally stopped by adding 100 μl of 2N H₂SO₄. The absorbance was measured using a synergy-4 plate reader at 450/630 nm. Finally, the Stimulation Index (SI) of the experimental mice was calculated using: (OD of stimulated wells-Blank)/(OD of un-stimulated wells-Blank).

Quantitative IL-4 and IFN-γ ELISA

Two weeks after the last immunization, 3 × 10^6 spleen cells per milliliter were re-suspended in complete RPMI 1640, placed in each well of a
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24-well plate and stimulated with 5 μg/ml of HBsAg for 72 h at 37°C in 5% CO₂. The supernatants were then collected and assessed for IFN-γ and IL-4 cytokines using a commercial ELISA Kit (Quantikine, R&D Systems, US) according to the manufacturer's manual.

ELISA of specific total antibodies and IgG1, IgG2a, IgG2b and IgM isotypes

The specific total antibodies were evaluated using an optimized indirect ELISA method. Briefly, 100 μl of HBsAg (5 μg/ml) in PBS was coated in 96-well ELISA Maxisorp plates (Nunc, Naperville, IL) followed by an overnight incubation at 4°C. The wells were washed 3 times with PBS containing 0.05% Tween 20 (washing buffer) and blocked for 1 h at 37°C with 5% skim milk in PBS buffer (blocking buffer). Serial dilutions of sera from 1/100 to 1/12,800 were prepared in 1% PBS-BSA. After washing the wells, 100 μl of each dilution was added to each well in triplicate and incubated at 37°C for 2 h. Then, the wells were washed five times with washing buffer and incubated for 2 h at 37°C. After the incubation, the wells were washed five times with washing buffer and 100 μl of the 1/10,000 dilution of anti-mouse conjugated to horseradish peroxidase Horseradish Peroxidase (HRP) (Sigma) in Phosphate Buffered Saline with 1% Bovine Serum Albumin (PBS-BSA) was added and incubated for 2 h at 37°C. Later, the wells were washed five times with washing buffer, 100 μl of TMB substrate was added, and the plates were incubated for 30 min in the dark. The reaction was stopped using 100 μl of 2N H₂SO₄, and the color density was measured at A₅₆₀/₆₃₀ nm with an ELISA plate reader. To detect the specific IgG1, IgG2a, IgG2b and IgM subclasses, goat anti-mouse IgG1, IgG2a, IgG2b and IgM secondary antibodies (Sigma) were used.

Statistical analysis

ANOVA tests were used to compare the statistical significance among the experimental groups. Values of P<0.05 were considered to represent statistically significant differences. The results are presented as the mean ± S.D.

Results

Preparation and characterization of Se NPs

The size and shape of the fabricated Se NPs were confirmed by FESEM. An FESEM image of the prepared SeNPs is shown in the upper illustration in Fig. 1. The FESEM image shows that particle size range is below 100 nm with an average size of approximately 60 nm. The surface charge of the synthesized Se NPs was −23.8 as determined by Malvern Zetasizer.

Proliferation assay

To assess the lymphocyte proliferative response, splenocytes were re-stimulated in vitro with 5 μg/ml of HBsAg for 72 h, and the proliferation assay was performed using the BrdU method. In comparison to the PBS and vaccine control groups, a significant increase in the stimulation index of the lymphocytes was observed in the mice that received 200 μg of synthetic SeNPs (P ≤ 0.05). The administration of 100 μg SeNPs had some effect on lymphocyte proliferation, but this change was not considerable in contrast to the other groups (Fig. 2).

Cytokine assay in spleen cell culture

IFN-γ and IL-4 were measured using a quantitative ELISA method. The administration of synthetic SeNPs at doses of 100 μg and 200 μg caused immunization in the mice and at a dose of 5 μg of
HBsAg vaccine the IFN-γ secretion was significantly increased in comparison with the PBS and vaccine control groups ($P < 0.01$). The amount of IL-4 level was the same in all of the groups, but the ratio of IFN-γ to IL-4 was higher in the groups that received 100 and 200 μg doses of SeNPs (Fig. 3). The result demonstrates the potential of the administration of synthetic SeNPs to viably shift the immune response toward the Th1 type.

**Humoral immune response and isotyping**

To obtain specific humoral immune responses with an optimized indirect ELISA method, initially 8 serial dilutions from all sera (collected 2 weeks after the last immunization) were prepared ($1/100$–$1/12,800$), and then the total antibodies were evaluated. The results showed an increase in the level of total antibody in the serum of the mice that received 100 and 200 μg doses of synthetic SeNPs plus HBs antigen vaccine in comparison to the PBS and vaccine control groups ($P \leq 0.05$) (Fig. 4). The results of the antibody isotype measurement showed that the IgM level rose significantly in mice that received 100 μg SeNPs in comparison to the vaccine and PBS control groups ($P \leq 0.05$). Meanwhile, the levels of IgG1 and IgG2b antibody in the 100 and 200 doses of SeNPs were significantly higher in comparison to the PBS control group (Fig. 5).

**Discussion**

Vaccination against Hepatitis B infection is the only assured option to prevent the spread of this disease, and the success of the vaccination approach using recombinant HBsAg was confirmed by the global health initiative to minimize the prevalence of this infection [2]. However, non-responsiveness to the vaccination has been widely described in some individuals, such as hemodialysis patients, diabetic individuals and the neonates of hepatitis B carrier mothers.
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Several studies have been previously designed to increase vaccine efficacy, especially by altering the vaccine composition and its delivery routes \[10\]. In the present study, the oral administration of SeNPs as a supplement during the vaccination schedule led to an increase in the level of IFN-\(\gamma\) and its ratio to IL-4 (Fig. 3C). The importance of IFN-\(\gamma\) in the immune response against Hepatitis B has been previously reported. Meanwhile, in another study, it was revealed that the IFN-\(\gamma\) content produced by peripheral blood mononuclear cells (PBMCs) after phytohemagglutinin (PHA) and HBsAg stimulation was lower in non-responders in comparison with responders \[11\].

IFN-\(\gamma\) is known as a Th1 network cytokine and plays a key role in promoting the T cell response. Both human and animal studies have demonstrated that induction of T cell responses is critical for the control of many viral infections \[12\]. A robust T cell immune response is also promoted at the acute phase of hepatitis B infection. IFN-\(\gamma\) has a fundamental role in inducing the natural killer (NK) cell response, also known as the first line of immune response against cancer and viral infection.

**Figure 4** Total IgG antibody in the experimental groups. Two weeks after the final immunization, the sera of the experimental mice were collected and the total antibody level was evaluated with an optimized indirect ELISA method. The data from each group are presented as the mean \(\pm\) SD (\(n = 15\)) (\(P \leq 0.05\)).

**Figure 5** Specific IgM, IgG1 and IgG2a isotypes in the immunized groups. The sera of the experimental mice were collected two weeks after the final booster and the specific IgM, IgG1 and IgG2a were evaluated by indirect ELISA as mentioned in method section. The results are presented as the mean \(\pm\) SD of each group (\(n = 15\)) (\(P = 0.003\)).
We have previously demonstrated that administration of biogenic SeNPs leads to a Th1-type immune response. In this study, the synthetic form of SeNPs caused the same results for the induction of cytokine production [9]. In the biogenic form of SeNPs, the surface of the nanoparticles may be changed due to residues from bacterial cytoplasm components like DNA or lipoproteins, and these elements probably are involved in triggering the immune response. Synthetic SeNPs reduced by ascorbic acid at the nanoparticle surface did not have the same residual elements. The results of the present research confirmed that the orchestration of the immune cells is only due to the incorporation of SeNPs. Meanwhile, considering the results of the BrdU test in the current work, our hypothesis was confirmed, which was that synthetic SeNPs had the ability to stimulate the immune cells in both proliferation as well as the production of cytokines. Cytokines play a major role in the regulation, differentiation and maturation of T and B lymphocytes. Despite the obtained IL4 measurements in the present study exhibiting no differences between the treatment and control groups, it was observed that the total antibody responses were elevated in mice that received both 100 and 200 μg of SeNPs in comparison to the vaccine and PBS control groups. Meanwhile the ratio of IFN-γ to IL-4 as an important index of Th1/Th2 response in the present work showed the priority of the IFN-γ response among the treated mice in comparison to the controls. Thus, the results revealed the capacity of the SeNPs to potentiate not only the humoral branch (B cells) but also the cellular branch (T cells) of the immune system.

However, the antibody isotype results indicated that only the IgM isotype was significantly raised in the mice administered 100 μg doses of SeNPs compared to the control vaccine. Surprisingly, the elevation of cytokines and total antibodies was slightly higher at the 100 μg dose compared to the 200 μg dose of SeNPs. This difference signified the necessity to carry out a detailed investigation of the dose response relationship of synthetic SeNPs. It is important to mention here that several researchers have proposed that antibody isotype and cytokine profiles do not always match [13–15]. Therefore, the differences among the IgG1, IgG2a and IgG2b levels in the SeNP-administered mice in comparison to the control vaccine as observed in the present study failed to increase the IFN-γ level. Moreover, as far as a significant rise in the IgM level due to the SeNP administration in comparison to the control vaccine was concerned, it may be hypothesized that for antibody class switching, in addition to the elevation of IgG classes of antibody that are proportionally related to the IFN-γ level, an increased time period, i.e., over 15 days or more, is required.

The mechanism underlying the non-responsiveness to hepatitis B surface antigen (HBs) in humans is largely unexplained, although evidence has shown that different HLA-DR alleles are associated with specific low responsiveness in different ethnic populations [16]. Regardless of the precise reason for the non-responsiveness, any approach to successfully improve the immune response after vaccination against hepatitis B infection seems worthy for the health surveillance system of any nation.

Conclusion

The results of the present research show that, because SeNPs are a new adjuvant in their infancy stage, they may be a good candidate not only for more specific studies in the aforementioned context but also to design a new preventive strategy with different administration routes for HBV vaccine studies.

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Competing interests

The authors declare that they have no conflict of interest.

Ethical approval

In this study all experimental procedures using animals was carried out according to the Animal Care and Use Guidelines of Tehran University of Medical Science (Tehran, Iran).

Authors’ contributions

Mehdi Mahdavi, Mohammad Hossein Yazdi and Hura Hashemi contributed in immunoassay experiments and SeNPs preparation. Ramin Farhoudi, Elnaz Faghfuri, Faranak Mavandadnejad, Somayeh Homayouni-Oreh collaborated in animal study and vaccination of animals. Ahmad Reza Shahverdi
designed the project and prepared the manuscript and analysis of data.

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