Research Paper:
Synthetic Nano-selenium Improving Macrophage Immune Responses Treatment of Bladder Tumor Antigens

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ABSTRACT

Background: Synthetic nanoparticles are deemed to improve treatment with the least adverse effects. The effect of Selenium Nanoparticles (SeNPs) was reviewed on various pathogenic disorders. In the present project, the role of SeNPs on macrophage responses was assessed.

Materials and Methods: SeNPs were prepared synthetically by ascorbic acid. Macrophages (MQs) were cultured and treated with SeNPs in combination with bladder tumor lysate and Bacillus Calmette Guerin (BCG). Other experimental groups include SeNPs + tumor lysate + MQs, BCG, BCG+MQs, and MQs. The mRNA levels of interferon-γ and interleukin-10 were evaluated using the real-time PCR method.

Results: Synthetic selenium nanoparticles combined with the tumor lysate upregulated the mRNA level of interferon-γ after 12 and 24 h treatment. Regarding interleukin-10 expression, there were no remarkable differences in all experimental groups. The maximum effect of synthetic SeNPs was observed after 24 h treatment.

Conclusion: The optimum effect of synthetic SeNPs presents in a treatment-dependent manner.

1. Introduction

Selenium is generally known in the field of nanobiology, particularly nanomedicines. Nanomedical approaches are applied for drug delivery, anti-inflammation, immunomodulation, and anti-cancer therapy [1]. Selenium generally presents in foodstuffs for humans, promotes glutathione peroxidase, and prevents free radicals from damaging cells in vivo [2, 3]. Owing to the Selenium Nanoparticles (SeNPs) biological features, the investigations in this field have been performed more recently [4, 5]. There are various approaches to nanoparticle synthesis. The primary methods comprise physical, chemical, biogenic, and synthetic. In the last decades, physical and chemical methods were the main types of nanoparticle fabrication; however, the physical method was expensive and consumed high energy...
Also, hazardous substances are utilized, and the production of destructive by-products is present in the chemical approach, which are important reasons for limiting the generation of pharmaceutical productions [11]. Much research has been performed on synthesizing nanoparticles with a safe and inexpensive method, a complementary method substituting the chemical approach. In this regard, some agents can perform synthetic preparation of SeNPs, by reducing properties such as lactose and ascorbic acid. Therefore, selenium dioxide can be reduced, and synthetic SeNPs would be prepared in the presence of reducing substances [12]. Thus, the synthesis of nanoparticles using synthetic methods can be considered a replacement strategy for the chemical and physical approaches. No harmful effects have been observed by synthetic fabrication based on published reports for physical and chemical methods. In this regard, bacteria can synthesize the metal nanoparticles such as copper, gold, selenium, and silver, which could be applied for anti-infection, immunotherapy, and anti-cancer medications [13].

Furthermore, Nanoparticles Synthesized by Metals (NSMs) have favorable characteristics, including antimicrobial, anti-cancer, and pharmaceutical features. The synthesis with high purity is an essential factor for metal-nanoparticles [13-16]. As explained above, nano-biotechnology is utilized in immunotherapy and medicines.

Various studies have been demonstrated that SeNPs have anti-cancer properties by suppressing the cell cycle, inducing apoptosis pathways, and reinforcing the immune responses [17]. Notably, bladder cancer, the fourth most common cancer, especially in men worldwide, could be treated by synthetic SeNPs [18]. As we know, BCG installation has been using for Non-Invasive Muscle Bladder Cancer (NIMBC). Although BCG therapy is partly successful for treating NIMBC, adverse effects and recurrence in these patients have been reported [19]. Therefore, due to their anti-cancer and safe features, synthetic SeNPs are comparable to BCG regarding the therapeutic effect of BCG. In this regard, the immune responses are the most crucial section in counteracting tumor cells [20]. MQs, as the vital component of the immune system, have anti-cancer activities [21].

Based on the related research, macrophages can enhance the effect of BCG instillation in bladder cancer via phagocytosis of BCG. Hence, activated macrophages can gain effector function. Many research studies have reported the inducer activity of BCG on macrophage cytotoxicity against mouse bladder tumors [22]. In this project, the effect of synthetic SeNPs is assessed on the macrophages immunity by Interferon-γ (INF-γ), macrophages activator, Interleukin-10 (IL-10), a suppressor cytokine of the immune system, which has an essential role in the inhibition of immune responses and are secreted from Tumor-Associated MQs (TAMs). IL-10 is the main cytokine produced from regulatory T cells (Tregs) and serves towards tumor progression. As well, IFN-γ is identified as cytokine profile of Th-1 and increases tumor phagocytosis by MQs, and presents tumor antigens to CD8+ cytotoxic T cells (CTLs) [23-26]. The effect of synthetic SeNPs on the gene expression of IFN-γ and IL-10 was evaluated in MQs and bladder tumor lysate compared to BCG immunological effect. The mRNA expression of these cytokines representing the macrophage responses when encountering bladder tumor lysate. MQs have a great capacity in triggering the innate and adaptive immunity against cancer progression [27-30]. Therefore, the evaluation of these cytokines can partly clarify the effect of SeNPs on macrophage responses and suppression of bladder cancer.

2. Materials and Methods

Cell line culture

EJ138 cell line was purchased from Pasteur Institute, Tehran, Iran. These cells were cultured in RPMI-1640 medium (Fetal Bovine Serum [FBS] 10%, streptomycin 100 mg/mL, penicillin 100 U/mL, L-glutamine 2 mM).

Tumor lysate preparation

We used the freeze-thaw method to obtain the tumor lysate based on the previous study [31]. Initially, FBS proteins were removed by culturing the tumor cells in serum without medium for 48-72 h. The cells were then washed twice in Hank's Balanced Salt Solution (HBSS) buffer (Gibco, USA) and lysed by the freeze-thaw method 5 times. The freezing process was conducted on dry ice, and the methanol thawing procedure was performed at room temperature. Next, the staining was performed by trypan blue, and the cellular disruption was evaluated using an inverted microscope. The tumor lysates were then sonicated for 12 min and centrifuged for 30 min (15000g at 4°C). The given supernatant was centrifuged (1000g at 4°C) for three hours.

Preparation of Selenium Nanoparticles (SeNPs)

Synthetic SeNPs were gifted from Dr. Mohammad Hossein Yazdi at Tehran University of Medical Sciences. The size of SeNPs was less than 35 nm.
**Preparation of sample and mononuclear cells culture**

Ten microliters of blood were drawn voluntarily from three healthy participants. We obtained consent from all participants in this study. The samples were diluted with 1/2 PBS and dispensed on the Ficoll-Hypaque density gradient. The buffy coat layer was isolated. After that, the staining of trypan blue was utilized for counting the cells. The adjustment of cell suspension was performed to $3 \times 10^7$ cell/mL, and 1 mL of cell suspension was added to culture dishes. To attach monocytes, the dishes were incubated at 37°C with 5% CO$_2$ for 2 h. Then, non-adherent cells were removed, and the dishes were rinsed 3 times to discard all non-adherent cells. The attached cells were deemed macrophage cells and stimulated by 100 µg/mL of SeNPs and 200 µg/mL of bladder tumor lysate (according to the previous study) [32] for 12 and 24 h. Macrophage cells were treated with 1 mg/mL of bladder tumor lysate (based on the study’s primary setup) and 100 µg/mL of SeNPs for 12 h and 24 h. Macrophage cells are considered negative, and BCG as the positive control group. Notably, monocytes are differentiated to macrophages under in vitro conditions. These five experimental groups are included as follows: 1) synthetic selenium nanoparticles in combination with tumor lysate and MQs (MQ/T+SSeNPs), 2) synthetic selenium nanoparticles in combination with tumor lysate, MQs, and BCG (BCG+MQ/T+SSeNPs), 3) MQ+tumor lysate, 4) MQ+BCG, and 5) MQs.

**Gene expression assay**

The expression level of mRNA of IFN-$\gamma$ and IL-10 cytokines were measured quantitatively by the real-time PCR method. The isolation of RNA was performed after 12 h and 24 h stimulation by RNA isolation kit (according to the manufacturer’s construction) (Yekta tajhiz, Iran), and cDNA was transcribed by RevertAid™ First Strand cDNA Synthesis Kit (Yekta tajhiz, Iran). Briefly, 5x solution buffer (4 µL), separated RNA (1 µg), deoxyribonucleotide triphosphate mixture (1 µL), random hexamer primer (1 µL), RNase enzyme inhibitor (1 µL), reverse transcriptase enzyme (1 µL), and double-distilled water (up to a final volume of 20 µL) comprised the cDNA synthesis process. The temperature program was planned as follows: The temperature was 24°C for 6 min, 45°C for 60 min, and 72°C for 6 min. The primers sequence of IFN-$\gamma$, IL-10, and $\beta$-actin (reference gene) is presented in Table 1. Consequently, the real-time PCR reaction was performed by a real-time PCR system (Applied Biosystems, Step One) at a temperature program of 95°C for 1 min, 95°C for 20 s, and 57°C for 60 s. Primers for IFN-$\gamma$ and IL-10 were applied at 10 nM. The given $\Delta\Delta$CT was utilized to analyze the mRNA expression, and $\beta$-actin was deemed as a normalizing gene. The primer sequences are brought in Table 1.

**Statistical analysis**

The mean value of each triplicate was assessed, and the finding of each immunoassay was expressed as mean±SD. The analysis of data was done in GraphPad prism V6.01 software. The Mann-Whitney U test was used to compare the statistical significance among the experimental groups. P values less than 0.05 indicate statistically significant differences between the experimental groups.

### 3. Results

#### Gene expression assay

**Twelve hours**

After the treatment, the IFN-$\gamma$ cytokine results showed a 2.83-fold increment in the BCG+MQ/T+SSeNPs experimental group compared to the MQ control group ($P<0.0001$). The experimental group treated with MQ/T+SSeNPs showed a 2.35-fold increment in comparison to the MQs, and both BCG+MQ/T+SSeNPs and MQ/T+SSeNPs experimental groups increased significantly versus the MQ+BCG ($P<0.0042$) and MQ+tumor lysate ($P<0.0068$) control groups. The MQ+BCG and MQ+tumor lysate significantly increased versus the MQ control group ($P<0.0001$) (Figure 1).

Results of IL-10 did not show a remarkable difference in the BCG+MQ/T+SSeNPs group compared to other experimental and MQs control groups. Also, other experimental groups did not have remarkable differences from each other ($P=0.99$) (Figure 2).

**Twenty-four hours**

The BCG+MQ/T+SSeNPs experimental group showed a 3.89-fold increase in IFN-$\gamma$ expression than the MQ control BCG group ($P<0.003$) increased IFN-$\gamma$ remarkably versus the MQ control group ($P<0.0001$). Also, the mRNA level of IFN-$\gamma$ demonstrated a remarkable elevation in the BCG+MQ/T+SSeNPs versus other experimental groups ($P<0.0001$). Also, MQ+tumor lysate ($P<0.0001$) and MQ+BCG group ($P<0.003$) increased IFN-$\gamma$ remarkably versus the MQ control group (Figure 3).

The results obtained from IL-10 expression could not show any significant differences in the BCG+MQ/
T+SSeNPs experimental group compared to other experimental and MQ control groups. Also, other experimental groups did not have remarkable differences from each other (P=0.99) (Figure 4).

4. Discussion

Various studies have demonstrated the high toxicity of Metalloid Nanoparticles (MNPs) in various cancers, including bladder, breast, etc. NSMs can also inhibit the growth of tumor cells via different cellular and molecular pathways such as cell cycle suppression, apoptosis induction, and disruption of cellular homeostasis [33-36]. The preparation of synthetic SeNPs has some advantages in comparison to other chemical and physical approaches. The lower toxicity of synthetic SeNPs is significant compared to fabricated SeNPs by chemical and physical approaches. The chemical and physical approaches are not environmentally friendly, and their hazardous by-products are not created in the synthetic approach. Besides, the synthetic approach for SeNPs preparation is more safe and effective than physical and chemical approaches. Eventually, the photoelectric and semiconducting properties are observed in synthetic SeNPs. The reduction of selenium to elemental selenium

| Table 1. Primer sequences |
|---------------------------|
| **Gene** | **Forward Sequence** | **Reverse Sequence** |
| IFN-γ | 5'-TTCTTACAACAAAAATCAAATCT-3' | 5'-TTCTTACAACAAAAATCAAATCA-3' |
| IL-10 | 5'-GCAATCCTCCTCAAGTAA-3' | 3'-TGAAGGATCGCTGACAAC-5' |
| β-actin | 5'-GAGGCGGCTACAGCTT-3' | 3'-TCCCTTAATGTCACGCATTT-5' |

![Image of Table 1](image)

**Figure 1.** Interferon (IFN)-γ mRNA level significant increase in synthetic SeNPs combined with MQ, tumor lysate and BCG versus other experimental and control groups. IFN-γ gene expression was measured using the real-time PCR method. P less than 0.05 is considered significant.

![Image of Figure 1](image)

**Figure 2.** Interleukin (IL)-10 expression unchanged in all experimental groups. IL-10 gene expression was measured using the real-time PCR method. P<0.05 is considered significant.

![Image of Figure 2](image)
The anti-tumor features of MNPs, particularly SeNPs, highlight their significant biological methods [37-39]. Also, regarding the adverse effects of BCG and lack of high efficiency in treating patients who experienced recurrence, the alternative therapeutic options should be investigated to replace the conventional therapies entirely or utilize them as complementary medicines in these patients [40, 41]. In the present study, the adjuvant effect of synthetic SeNPs on macrophage responses was evaluated from two perspectives. Firstly, macrophages are the professional phagocytic cells, particularly in exposure to tumor cells. Secondly, there is a significant correlation between macrophage activity and BCG in bladder tumor cells based on various research studies [42, 43].

Furthermore, macrophages isolated from healthy people are mainly M1 type and release some particular cytokines such as IFN-γ, Tumor Necrosis Factor-α (TNF-α), and IL-12. Macrophages can kill the pathogens and tumors by themselves (directly) or act as antigen-presenting cells (indirectly). Also, tumor antigen presentation to T cells can induce adaptive responses. Therefore, macrophages are pivotal to encounter against cancerous cells [44, 45]. This research indicates that synthetic SeNPs on their own and combined with the MQ, tumor lysate, and BCG can remarkably elevate the IFN-γ expression compared to the non-treatment control group. Yazdi et al. have shown the critical effect of SeNPs on pro-inflammatory cytokines, including TNF-α and gamma interferon levels [32]. In another study, Yazdi et al. demonstrated that SeNPs potentiated immune responses in the breast tumors by raising the serum level of IL-2, IL-12, and IFN-γ [46]. Besides, IL-27, IFN-γ, and IL-12 are secreted through activated macrophages against tumor cells. The anti-tumor performance of these cytokines has been demonstrated in multiple studies [47, 48]. As we have known, IL-10 is considered an anti-inflammatory cytokine that facilitates tumor progression. Herein, our results demonstrated that the fold expression level of IL-10 was not significant among experimental groups compared to each other. Cardillo et al. have shown that IL-10 expression was increased in patients with bladder cancer [49].
We found that the synthetic SeNPs combined with macrophages and BCG could suppress IL-10 gene expression, and then the growth of tumor cells would be confined. IL-10 downregulation represented the reinforced anti-tumor effects of synthetic SeNPs along with BCG and MQs because of having synergistic effects in these molecular interactions. MQs increase tumor cells’ presentation to adaptive immunity to kill the cancerous cells. Besides, BCG has multiple anti-tumor biological functions, and studies showed that direct instillation of BCG could increase leukocytes drastically in the urine. Also, BCG can release different inflammatory cytokines and chemokines (IFN-γ, TNF-α, Granulocyte-Macrophage Colony-Stimulating Factor [GM-CSF], IL-1, IL-5, IL-2, IL-8, IL-6, IL-18, IL-12) into the bladder, and it affects priming and recruiting T cells to the bladder [50, 51]. Biot et al. have indicated T cells’ function in the anti-tumor performance of BCG and shown that activating T cells in response to BCG treatment occurs in the para-aortic lymph nodes [52]. Other studies showed that the infiltration of Natural Killer cells (NKs) and T cells is increased in the bladder cancer tissue following BCG therapy [53]. Besides, MCH-II and ICAM-1 expression are increased by BCG therapy [54]. Our results showed that BCG and MQs, along with tumor lysate, promoted the macrophage responses of EJ tumors of bladder cancer.

Overall, synthetic SeNPs applied in this project displayed a suitable potency to reinforce macrophage responses by elevating the mRNA expression of the most critical cytokines in the adaptive immunity, i.e., IFN-γ, and reducing IL-10. Actually, adaptive and innate immunity were potentiated through elevating IFN-γ expression, and tumor mediated response has been declined through decreasing IL-10 expression.

Furthermore, our findings represented that synthetic SeNPs have a synergistic activity and bladder tumor lysate because of upregulated expression levels of IFN-γ. So, it could cause the antigen presentation and anti-tumor activity of macrophage cells.

5. Conclusion

The findings reveal that the bladder tumor lysate improves the effect of synthetic SeNPs on the induction of macrophage cells’ immune responses. The maximum expression level of IFN-γ was obtained when SeNPs were combined with the bladder tumor lysate, BCG and MQs as it was concluded that synthetic SeNPs could be a promising option for complementary medication.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors’ contributions

Conceptualization: Mohammad Hossein Yazdi; Software, validation, formal analysis: Setareh Haghighat; Investigation: Mohammad Hossein Yazdi and Zeinab Agharezaie; Writing: Zeinab Agharezaie; Review and edit: Mohammad Hossein Yazdi and Setareh Haghighat.

Conflicts of interest

The authors declared no conflict of interest.

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