EVALUATION OF SELENIUM NANOPARTICLES AS A POTENTIAL CHEMOPREVENTIVE AGENT AGAINST LUNG CARCINOMA

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ABSTRACT:
Selenium nanoparticles (Se-NPs) have garnered a great deal of attention as potential cancer therapy. The use of microorganisms in the synthesis of nanoparticles emerges as an eco-friendly and exciting approach. This study was designed to assess Se-NPs synthesized by Bacillus Licheniformis as chemotherapeutic agent against lung cancer induced by ferric-nitrilotriacetate (Fe-NTA).
Se-NPs were synthesized through the reduction of aqueous selenium ion using the growth culture supernatants. This method is capable of producing Se-NPs in a size range of about 50-80 nm and characterized by transmission electron microscopy. Thirty six male rats were divided into four groups as follows: Group 1: control, Group 2: oral administrated with Se-NPs 0.2 mg Se/kg body weight, Group 3: intraperitoneal injected with Fe-NTA, Group 4: pretreatment with Se-NPs then injected with Fe-NTA with contentious administration with Se-NPs. Treatment with Fe-NTA caused significant reduction in glutathione level, glutathione peroxidase, catalase and superoxide dismutase activities with marked elevation in lipid peroxidation, nitric oxide, C-reactive protein and TNF-α level. Lung tissue histological analysis of Fe-NTA treated rats showed islands of hyperplasia cells. Pretreatment with Se-NPs significantly restored glutathione level, glutathione peroxidase, catalase and superoxide dismutase activities and ameliorated oxidative damage parameters of lipid peroxidation, nitric oxide, and inflammation parameters C-reactive protein and TNF-α level with noticed improvement in the pulmonary tissue histological analysis and disappearance of hyperplasia cells.
Microbial synthesized Se-NPs shows promising era in the management of lung cancer as Se-NPs was able to: restore antioxidant state; reduce oxidative stress, inflammation accompanied with disappearance of hyperplasia in lung tissue.

KEYWORDS:
Lung, cancer, selenium nanoparticles, ferric-nitrilotriacetate.

INTRODUCTION
Worldwide, lung cancer remains the leading cause of cancer death. Decades of smoking and other environmental and occupational risk factor in older adults will continue to produce millions of new cases of lung cancer worldwide. Several studies have reported reduced risks of lung cancer in people in the higher categories of selenium intake, although other studies have failed to confirm this association, [1, 2, 3].
There is an increasing evidence that cancer and other mutation-related diseases can be prevented not only by avoiding exposures to recognized risk factors but also by favoring the intake of protective factors and by modification of defense and DNA repair mechanisms of the host organism. Selenium, an essential trace element, belongs to the most extensively studied chemoprevention compounds. Some human ecological studies have reported associations between low selenium intake and increased cancer mortality. Selenium deficiency is related to the occurrence of specific chronic diseases, including cancer in humans, while adequate selenium intake is associated with the lower incidence of certain cancers in humans. [4]. Selenium is a necessary trace element in mammalian and human. It is well supported that selenium has chemopreventive effects, and emerging evidences suggest that selenium has chemotherapeutic potential by inducing cancer cell apoptosis with minimal side effects to normal cells within a proper dose range. Selenium induces G2/M cell cycle arrest and apoptosis in colorectal cancer cells via mitochondrial pathway, [5]. The mechanisms of apoptosis for selenium compounds
mainly involves a mitochondrial pathway, protein kinases, tumor necrosis factor, activation of caspases and reactive oxygen species, [6].

Nanotechnology plays a very important role in many key technologies of the new millennium. The application of nanoscale and nanostructure materials within the range of 1 to 100 nm is an emerging area of nanoscience and nanotechnology. Nanomaterials may provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine, and water treatment, [7].

The prospect of natural resources for metal nanoparticles synthesis has become to be a competent in the perspective of cost effective and eco-friendly points. Researchers prefer a biological synthesis because the distribution control of particles obtained from this method is better than other methods, [8]. Nanoparticles may be synthesized either intracellularly or extracellularly employing yeast, fungi bacteria or plant materials. The biological synthesis of nanoparticles is highly eco-friendly and possesses distinct advantages such as enhanced stability, better control over the size, shape, and monodispersity of the nanoparticles, when compared with the more traditional physical and chemical methods which often involves the use of hazardous chemicals creating environmental concern. Metal nanoparticles have received significant attention in recent years owing to their unique properties and practical applications,[9]. Nanoparticles are biosynthesized when the microorganisms grab target ions from their environment and then turn the metal ions into the element metal through enzymes. The intracellular method consists of transporting ions into the microbial cell to form nanoparticles in the presence of enzymes. The extracellular synthesis of nanoparticles involves trapping the metal ions on the surface of the cells and reducing ions in the presence of enzymes, [10].

The work of the test was to evaluate the efficacy of Se-NPs with further insight into the possible mechanisms by which selenium may impact lung cancer risks. We employed an animal model of lung carcinoma that is induced by oxidative damage via Fe-NTA to examine Se-NPs synthesized by bacteria as cancer protective agent.

**MATERIAL AND METHODS**

**Preparation of Fe-NTA solution**
The Fe-NTA solution was prepared according to, [11]. In brief, disodium salt of nitritriacetic acid (NTA) (0.64 mmol/kg body weight) and ferric nitrate enneahydrate (0.16 mmol/kg body weight) were dissolved in distilled water, and the pH was adjusted to 7.0 using sodium bicarbonate. The molar ratio of Fe to NTA was 1:4. Nitritriacetic acid (NTA) is used as polyphosphate substitute in detergents in various countries; it forms water-soluble chelate complexes with metal cations at neutral pH.

**Biosynthesis of selenium nanoparticles**
*Bacillus licheniformis* strain (*B. licheniformis*) ATCC 10716, was obtained from Microbiological Resources Centre (Cairo MIRCEN). 2g of wet *B. licheniformis* biomass was taken in an Erlenmeyer’s flask. 1mM SeO₂ solution was prepared using deionized water and 100 mL of the solution mixture was added to the biomass, and kept in a shaker at 37°C (200 rpm) for 24 h for the synthesis of nanoparticles. Finally, the resulting solution was filtered through a 0.22 µm filter (Millipore), [12].

**Transmission electron microscopy analysis**
Synthesized Se-NPs by *B. licheniformis* were analyzed by transmission electron microscopy (TEM). Samples of Se-NPs were prepared by placing a drop of the suspension of Se-NPs on carbon-coated copper grids and allowing water to evaporate. The shape and size of nanoparticles were determined from TEM micrographs. The software (Advanced Microscopy Techniques, Danvers, MA) for the digital TEM camera was calibrated for size measurements of the nanoparticles. TEM measurements were performed on a JEOL model 1200EX, [13]. For ultrastructural studies, 24 h old culture grown in the presence of 2 mM selenium dioxide and from control were centrifuged at 1844 × g for 15 min, [14].

**Animals and experimental design**
Male Wister rats weighing about 120-150 g were provided by the Animal House of the Nile Company for Drugs, Cairo, Egypt. Rats were housed in plastic cages and maintained under standard laboratory conditions (temperature 25 ± 2°C; photoperiod of 12 h) on a commercial pellet diet and water ad libitum.

Toxicological studies of Se-NPs: A 30-day toxicity study of Se-NPs was conducted in male rats by studying serum biological markers, survival and decrease in body weights. Sixty male rats were divided into sex groups of ten animals each. Five groups were given Se-NPs of 50, 100, 200, 400 and 500 µg Se/kg, once daily for 30 days. One group was served as control and given sterile physiological saline. After a week of acclimation, thirty four male rats were randomly divided into four groups of eight animals each, group 1: control, group 2: orally administrated with Se-NPs, 3 times a week (each alternate day) in a dose of 200 µg Se/kg body weight, group 3: injected i.p. with Fe-NTA twice weekly (9 mg/kg body weight), group 4: pretreated with Se-NPs for 15 days prior to Fe-NTA injection, the animals were sacrificed after 6 months.
Biochemical analysis
Antioxidant parameters were measured in the liver homogenate (10 % in 0.9 % saline). Glutathione (GSH) content was measured calorimetrically according to the method described by, [15]. Glutathione peroxidase (GPX) activity was measured calorimetrically according to the method described by, [16]. Superoxide dismutase (SOD) activity was measured according to the method described by, [17] and catalase activity (CAT) was measured calorimetrically according to the method described by, [18]. Lipid peroxides content was determined according to the method of, [19] using 1,1,3,3-tetra ethoxypropane as a standard. Nitric oxide was determined according to, [20].

Evaluation of tumor necrosis factor- alpha (TNF-α) in the plasma was carried out according to, [21], using the "Assay Max Rat TNF-alpha ELISA kit of murine monoclonal antibody" (ASSAYPRO, 41 Triad South Drive St. Charles, MO 63394, USA). Data was analyzed using 990win6 software for DV990BV4 microplate reader, GIO DE VITA, Roma, Italy. Determination of C-reactive protein was carried out by immunoturbidometry method according to, [22].

Histological analysis
Sections of mammary glands were stained with hematoxylin & eosin (H&E) and examined by light microscope, [23].

Statistical analysis
Statistical analysis was carried out using Excel and Results were represented as mean ± SD. SPSS version 15. P value ≤ 0.05 was considered to be statistically significant.

RESULTS
Studying transmission electron microscope (TEM) photos of B. licheniformis showed morphological changes in bacterial cells caused by exposure to selenium dioxide. Non-treated B. licheniformis cells showed rod shape while treated cells with selenium dioxide showed spherical shape Fig. 2. Photos showed accumulation of selenium inside and on bacterial cells as precipitations inside and on the bacterial cell. Characterization of the synthesized Se-NPs was carried out. The UV-Visible absorption spectra of selenium nanospheres recovered from the culture broth gave characteristic peak at 590 nm.

The morphology and size of the biologically synthesized Se-NPs was determined using TEM Fig.3. The size of Se-NPs was analyzed by TEM imaging which demonstrate that the prepared Se-NPs aggregated easily and their size was not uniform, ranging from 50 to 80 nm diameter. The morphological characterization of Se-NPs by TEM showed that Se-NPs is of spherical shape, depicts that they are relatively uniform in diameter and spherical in shape.

In vivo nanoparticles toxicity studies are focused mainly on examining changes in blood serum chemistry, animal population count and behavior. Thus the rats groups injected with Se-NPs at a dosage of 50, 100, 200, 400 and 500 µg Se/kg did not show any symptoms of toxicity such as fatigue, loss of appetite, change in fur color, weight loss, behavior change and serum chemical analysis of alanine amino trasferase, aspartate amino transferase, urea and creatinie. All the rats survived throughout the experimental period without exhibiting any abnormalities.

The effects of Se-NPs (200 µg Se/kg body weight) pretreatment on antioxidant state of Fe-NTA treated rat lung tissue. As Fe-NTA treatment significantly reduce measured antioxidant parameters of GSH level, GPx, CAT and SOD activities compared to the control. Pretreatment with Se-NPs caused significant elevation in GSH level, GPx, CAT and SOD activities compared to Fe-NTA, Table.1.

Examining the effect of Se-NPs pretreatment on Fe-NTA treated rats oxidative stress parameters lipid peroxidation (MDA) and nitric oxide (NO) results showed that Fe-NTA significantly increased MDA and NO levels, while pretreatment with Se-NPs markedly ameliorated MDA with non-significant reduction in NO level Fig.3,4. Inflammatory marker C-reactive protein (CRP) Fig.5 and tumor necrosis factor alpha (TNF-α) Fig.6 results revealed significant increase in CRP in Fe-NTA treated rats while pretreatment with Se-NPs significantly ameliorated this elevation in the plasma. CRP level significantly increased in Fe-NTA treated subjects as compared to the control. Pretreatment with Se-NPs markedly ameliorated CRC and increase compared to control.

The pathological effect of the Se-NPs and Fe-NTA over the morphological characteristics of the organs was examined through the histological observations using light microscope. Histological studies of control lung tissue showed normal pulmonary architecture, well formed air sacs or vesicles with normal and wide air passage also normal bronchial feature (Fig.7a). The examined reports obtained from the senior pathologist confirmed that the Se-NPs treated rat lung did not show any significant morphological changes in comparison to control. (Fig.7b). Lung sections of Fe-NTA treated rats clearly showed abnormal pulmonary architecture, a noticed destruction occurred in the intrapulmonary structure, very narrow and restricted alveolar ducts and destroyed alveolar vesicles are present. A great area was replaced by numerous islets of hyperplasia cells. These cells have been made a great invasion to the outer muscular layer of the bronchiole and surrounded
perialveolar and peribronchial areas (Fig. 7c). Pretreatment with Se-NPs of Fe-NTA treated rats showed a noticed improvement in pulmonary tissue structure, well alveolar sacs and ducts and well formed bronchiole but still some little inflammatory cells present peribronchiole, normal epithelial cells of the bronchiole with goblet cells and clear circular muscle layer around epithelium (Fig. 7d).

Fig. (1): Transmission electron microscope of selenium nanoparticles (20000x).

Fig. (2): Transmission electron microscope of B. licheniformis cells, A: control, B: exposed to selenium dioxide for 48 hr.

Fig. 3. Effect of Se-NPs against Fe-NTA induced alteration on lipid peroxidation level of lung.
Fig. 4. Effect of Se-NPs against Fe-NTA induced alteration on nitric oxide level of lung.

Fig. 5. Effect of Se-NPs against Fe-NTA induced alteration on CRP plasma level.

Fig. 6. Effect of Se-NPs against Fe-NTA induced alteration on TNF-α plasma level.
Fig. 7. Histopathological sections of lungs showing the effect of pretreatment of rats with Se and Nano-Se on Fe-NTA promoted lung carcinogenesis. (a) Control (H&E, 100). (b) Nano-Se treated (H&E, 100). (c) Fe-NTA treated (H&E, 100). (d) Se+Fe-NTA treated (H&E, 100).

Table (1): Effect of Se-NPs and Fe-NTA on glutathione, glutathione peroxidase, catalase and superoxide dismutase.

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH (mg/g)</th>
<th>GPx (µmol oxidized GSH/ min/g)</th>
<th>CAT(µM H₂O₂/g)</th>
<th>SOD (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.0± 2.2</td>
<td>171.0± 5.8</td>
<td>97.6± 4.6</td>
<td>12.1± 0.4</td>
</tr>
<tr>
<td>Se-NPs</td>
<td>27.1± 1.4a</td>
<td>169.7± 5.5</td>
<td>102.8± 6.1</td>
<td>11.8± 0.4</td>
</tr>
<tr>
<td>Fe-NTA</td>
<td>14.6± 0.8a</td>
<td>117.6± 6.8b</td>
<td>43.0± 3.9a</td>
<td>9.4± 0.3a</td>
</tr>
<tr>
<td>Fe-NTA+Se-NPs</td>
<td>19.0± 2.1ab</td>
<td>139.8± 8.4ab</td>
<td>95.9± 4.5ab</td>
<td>11.8± 0.5ab</td>
</tr>
</tbody>
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a: Significantly different from control, b: Significantly different from Fe -NTA group.

DISCUSSION

In the present study, Se-NPs synthesis by the strain B. licheniformis formed reddish cell suspension which indicated its ability to reduce the toxic, colorless, soluble selenium ions to nontoxic, red elemental insoluble form of selenium (Se⁰). Under the stressful condition of toxic selenium ions the morphology of the cells was altered resulting in decrease in cell size. The organisms reduce their cell size and increase their relative surface area for better uptake of the nutrients for survival under environmental stress conditions. Analyzing B. licheniformis cells exposed to selenium by TEM confirmed the spherical intracellular and extracellular deposits of SNPs. However, our data showed formation of intracellular and extracellular nanometer-sized particles of elemental selenium (Se⁰). Intracellular Se-NPs accumulation was inside the cytoplasm or periplasmic space of the bacterial cell as in TEM images and confirmed by other research groups, [24]. TEM confirmed the spherical shape and size range of 50-80...
nm. Nanoparticles having dimensions of the order of less than 100 nm have attracted great attention due to their unusual and fascinating properties, and applications advantageous over their bulk counterparts, [25].

In the present study, iron administration markedly reduced antioxidant markers GSH, GPx, CAT and SOD activities and inflammation markers CRP and TNF-α in the lung followed by tumor promotion. This is possibly due to generation of free radicals in the tissue forming FeCl₃ and •OH by a reaction of Fe-NTA with H₂O₂ in vivo might be involved in carcinogenesis, [26, 27]. Oxidative stress and inflammation are closely associated with tumor promotion, [28]. Iron deposition within tissue is often related to generation of reactive oxygen species, leading to oxidative damage, lipid peroxidation and concomitant increase in serum toxicity markers and depletion of renal GSH content, [29].

This is a unique experimental model, useful in search of antioxidant compounds by the pretreatment for 2 weeks and of chemopreventive compounds by oral administration for several months. We observed that oral pretreatment of Se-NPs protected lung tissue from iron ions induced damage. We demonstrated that Se-NPs inhibited elevation of oxidative injury markers of MDA and NO and maintained the levels of reduced GSH, GPx, CAT and SOD activities and ameliorated inflammation markers CRP and TNF-α in lung.

Selenium pretreatment may play roles to protect the living organism from iron ions-induced oxidative stress. Selenium remarkable biological effect in eukaryotes is related to a unique function of various selenoproteins by being incorporated into as selenocysteine and included such as glutathione peroxidase, thioredoxin reductase, manganese superoxide dismutase, cytosolic glutathione peroxidase and selenoprotein P which have antioxidant and detoxification functions. Selenoproteins family play critical role in cellular protection from oxidative damage and infection, male fertility, thyroid hormone metabolism and DNA synthesis [1,2,30,31].

Supra-nutritional levels of selenium have benefits in preventing several types of cancer, including lung cancer, colorectal cancer, and prostate cancer. It is well supported that selenium has chemopreventive effects, and chemotherapy potential by inducing cancer cell apoptosis with minimal side effects to normal cells within a proper dose range, [31,32,33,34,35]. However, the precise mechanisms by which selenium activates the apoptotic machinery remain poorly understood, [36]. In the present work, the cytokine TNF-α was highly elevated in Fe-NTA treated rats and markedly reduced by Se-NPs pretreatment. The cytokine TNF-α is highly expressed in tumors and has powerful and toxic systemic side effects, it is also a potent anti-vascular cytokine at higher doses and can be used clinically to destroy tumor vasculature. TNF-α is able to initiate cellular apoptosis and it is possible that these apoptotic pathways are deactivated in tumor cells, [37]. In the present work Se-NPs pretreatment significantly normalized CRP level. This liver synthesis protein, a marker of inflammation, can amplify the anti-inflammatory response through complement activation, tissue damage, and activation of endothelial cells and play a key role in the innate immune response, [38, 39]. Elevated plasma level of CRP is associated with increased risk of lung cancer, and possibly colorectal cancer and is associated with greater risk of cancer patient early death, but do not cause cancer, [40]. Plasma CRP levels decreased in the presence of antioxidant as Pycnogenol and vitamin C was reported, [41], and significantly associated with the biomarkers of oxidative stress malondialdehyde, [42]. The impact of antioxidants on plasma CRP may be mediated by effects on upstream cytokines, in particular interleukin-1 (IL-1), TNF-α, and interleukin-6 (IL-6), which are the main inducers of the acute phase response. In this study, Se-NPs inhibited the lipopolysaccharide-induced TNFα production, as well as CRP production, suggesting oxidative potential mechanism. Oxidative damage leads to an inappropriate activation of the transcription factor nuclear factor κB (NF-κB) and subsequently to an overexpression of inflammatory proteins. This potential oxidative mechanisms consistent with our observation in the present dataset that plasma TNF-α, and biomarker of oxidative stress MDA in lung tissue was significantly reduced by Se-NPs treatment accompanied with activation of antioxidant parameters GSH,SOD,CAT and GPx , [41].

In our study, histological/histopathological assays provide evidences over the morphological changes confirming biochemical studies, evidencing that toxicity correlate with changes in tissue and cell morphology. The histopathological findings of the non-toxic effect of the Se-NPs over lung were observed. Significant morphological changes induced by Fe-NTA implicated to iron ions oxidative stress damaging effects which probably cause changes in cellular DNA inducing hyperplasia cells appeared in TEM images. Pretreatment with Se-NPs ameliorated FE-NTA histological changes with disappearance of cancer cells as a result of protective effect of selenium on DNA, [43]. Supplementation of selenium compounds reduced cell proliferation in vitro, as well as a decreased incidence and severity of cancer in vivo , [44]. Selenium was reported to induce apoptosis via mitochondrial pathway.
dependent on caspase-3 activation regardless of P53, [45].

CONCLUSIONS

Based on these findings, we conclude that the aerobically produced Se-NPs by the microbe B. licheniformis, ATCC 10716 induces chemoprevention against lung cancer induced by iron ions via induction of antioxidant state and reduction of oxidative stress and inflammation markers of CRP and TNF-α. This study provides useful information for the use of Se-NPs to treat lung cancer.

REFERENCES


International Journal of Pharmaceutical, Biological and Chemical Sciences | e-ISSN: 2278-5191 | OCT-DEC 2013 | VOLUME 2 | ISSUE 4 | 38-46


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