Title:
Protective effects of sodium selenite supplementation against irradiation-induced damage in noncancerous human esophageal cells.

Authors:
Irma M. Puspitasari(1,2), email address: m12702053@gunma-u.ac.jp
Chiho Yamazaki(1), email address: kchiho@gunma-u.ac.jp
Rizky Abdulah(2), email address: abdulahrizky@gmail.com
Mirasari Putri(1), email address: putrimirasari@yahoo.com
Satomi Kameo(1), email address: skameo-t@gunma-u.ac.jp
Takashi Nakano(3), email address: tnakano@gunma-u.ac.jp
Hiroshi Koyama(1), email address: hkoyama@gunma-u.ac.jp

Affiliations:
(1) Department of Public Health, Gunma University Graduate School of Medicine, Gunma, Japan.
(2) Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, Bandung, Indonesia.
(3) Department of Radiation Oncology, Gunma University Graduate School of Medicine, Gunma, Japan.

Corresponding author:
Hiroshi Koyama, MD, PhD
Department of Public Health, Gunma University Graduate School of Medicine
3-39-22 Showa Machi, Maebashi 371-8511, Gunma, Japan
Tel: +81 27 220 8010; Fax: +81 27 220 8016
E-mail: hkoyama@gunma-u.ac.jp

Keywords:
Sodium selenite, radioprotection, X-ray irradiation, esophageal cells, apoptosis, irradiation-induced damage

Running title:
PUSPITASARI et al: PROTECTIVE EFFECTS OF SODIUM SELENITE AGAINST IRRADIATION-INDUCED CELL DAMAGE
Abstract

The administration of radioprotective compounds is one approach to prevent radiation damage in normal tissues. Thus, radioprotective compounds are important in clinical radiotherapy. Selenium is one radioprotective compounds that has been widely used in clinical studies of radiotherapy. However, evidence regarding the effectiveness of selenium use in radiotherapy and the mechanisms through which selenium reduces the side effects of radiotherapy remains insufficient. To provide further evidence of the effectiveness of selenium in radiotherapy, this study investigated the protective effects of sodium selenite supplementation prior to X-ray irradiation treatment in noncancerous human esophageal cells (CHEK-1). Sodium selenite supplementation increased glutathione peroxidase 1 (GPx-1) activity in a dose- and time-dependent manner. The sodium selenite dose that resulted in the most GPx-1 activity was 50 nM for 72 h. The half maximal inhibitory concentration (IC50) of sodium selenite in the cells was 3.6 µM. Sodium selenite supplementation increased the survival rate of the cells in a dose-dependent manner and increased cell viability at 72 h post irradiation (p<0.05). Combined treatment of 50 nM sodium selenite and 2 Gy X-ray irradiation decreased sub-G1 cells compared with 2 Gy X-ray irradiation alone from 5.9 to 4.2% (p<0.05) and increased G1 cells from 58.8 to 62.1%. Western blot analysis revealed that treatment with 2 Gy X-ray irradiation increased the expression level of cleaved poly ADP ribose polymerase (PARP) (p<0.05). In addition, combined treatment of 50 nM sodium selenite and 2 Gy X-ray irradiation reduced the expression of cleaved PARP protein compared with 2 Gy X-ray irradiation alone. Our results suggest that 50 nM sodium selenite supplementation for 72 h before irradiation can protect CHEK-1 cells from irradiation-induced damage by inhibiting irradiation-induced apoptosis. Sodium selenite is a prospective radioprotective compound for normal cells in clinical radiotherapy.
Introduction

Radiotherapy is one of the most common and effective treatments for cancer (1). Greater than 40% of cancer patients require radiotherapy during the management of their disease (2). Although clinical radiotherapy treatment planning and delivery technologies have improved, the toxicity of radiotherapy to normal tissues and organs remains a problem (2, 3). Thus, radioprotective compounds are very important in clinical radiotherapy (3) and the administration of radioprotective compounds has been suggested as one approach for preventing radiation damage in normal tissues (4, 5).

Selenium is a trace element that has fundamental importance in human biology (6). Selenium detoxifies reactive oxygen species (ROS) produced by irradiation treatment (4, 7). Selenium acts in human antioxidant systems in the form of selenocystein (SeCys), which is incorporated into various selenoproteins (8, 9). At least 25 selenoproteins have been identified in humans, including glutathione peroxidase (GPx), thioredoxinreductases (TrxR), iodothyroninedeiiodinase, and the selenoproteins P, W and R (10). In nature, selenium exists in many chemical forms. The most well studied forms are selenomethionine (SeMet), sodium selenite, selenium methylselenocysteine, 1,4-phenylenebis (methylene) selenocyanate (p-XSC), and methylseleninic acid (MSA) (9). Sodium selenite is the chemical form of selenium that was used for radiotherapy supplementation clinical studies between 1987 and 2012 (7).

Despite being broadly used as a complementary medicine during radiotherapy (11, 12), evidence regarding the effectiveness of selenium use in radiotherapy and the mechanism by which selenium reduces the side effects of radiotherapy remains insufficient (7). Schleicher et al (13) and Hehr et al (14) performed in vitro studies of selenium and radiotherapy and found that sodium selenite has potential as a protective agent for normal tissues during radiotherapy (15). However, the mechanisms of this protection have not been revealed. Diamond et al (16) reported that low level supplementation of culture media with selenium in the form of sodium selenite significantly protected CHO-AA8 cells, a hamster ovary-derived cell line, from radiation-induced mutagenesis. Eckers et al (17) also reported that overexpression of selenoprotein P (SEPP1) suppressed late radiation-induced ROS accumulation and protected normal human fibroblasts from radiation-induced toxicity. Tak and his coworker (18) found that when U937 cells, a human leukemic monocyte lymphoma cell line, were exposed to 2 Gy of γ-irradiation, a distinct difference was noted between cells that were or were not pretreated with ebselen with respect to apoptotic features and mitochondrial function.

However, more evidence is required to determine the mechanisms which selenium supplementation helps prevent the side effects of radiotherapy before it can be recommended as a cancer adjuvant radiotherapy. Given that sodium selenite is the only chemical form of selenium that has been used in clinical studies in this context, this study investigated the protective effects of sodium selenite supplementation on noncancerous human esophageal cells before X-ray irradiation.
Materials and Methods

Cell culture
Cells of the immortalized noncancerous human esophageal cell line CHEK-1 (19), were maintained in RPMI-1640 (Wako, Japan) with 10% fetal bovine serum (Hyclone, Utah, USA) and 1% penicillin-streptomycin (Gibco, New York, USA) at 37°C in a 5% CO₂ humidified chamber. The culture medium was replaced every 3 days, and the cells were passaged on a weekly basis using a 1:5 splitting ratio.

Selenium supplementation
Sodium selenite (Sigma, St Louis, USA) was the chemical form of selenium that was used for supplementation. The dose of sodium selenite supplementation ranged from 0-200 nM, and the time of incubation ranged from 24 to 72 h after 18 h of initial seeding. Supplementation with a dose of 50 nM for 72 h before irradiation treatment was used for the cell viability assay, cell cycle analysis and Western blot analysis.

Irradiation
Irradiation was performed using an X-Ray irradiation machine (Titan-225S, Shimadzu, Japan) at a rate of 1.3 Gy/min. The dose of irradiation was 2 Gy based on the common fractionation dose for radiotherapy.

Protein extraction for GPx-1 activity assay and Western blot analysis
CHEK-1 cells were supplemented with sodium selenite for 72 h, washed twice with phosphate-buffered saline (PBS) and harvested. Then, proteins were extracted using RIPA buffer (Sigma, St. Louis, USA) with 10% protein inhibitor (Sigma, St. Louis, USA). Protein concentrations were determined using a Bio-Rad DC protein assay kit (Bio-Rad, Tokyo, Japan) following the method of Lowry (20). The extracted sample was stored at -80°C until the day of analysis.

GPx-1 activity assay
The enzymatic activity of GPx-1 in CHEK-1 cell homogenates was determined using the method of Paglia and Valentine with some modification (21). GPx-1 activity was indirectly monitored using spectrophotometric methods to observe the reduction of oxidized glutathione using nicotinamide adenine dinucleotide phosphate (NADPH) as the reducing agent. GPx-1 activity was quantified by measuring the change in NADPH absorbance at 340 nm and was expressed as the change in NADPH absorbance (Δ mM NADPH) over time (min) and with different levels of protein (mg) in the presence of the substrate tert-
butyl hydroperoxide. Absorbance was observed using a microplate reader (SpectraMax Plus 384, Molecular Devices, CA, USA).

**Cytotoxicity assay**

Cytotoxicity of sodium selenite in CHEK-1 cells was examined with various concentrations of sodium selenite (0-8 µM) using a colorimetric assay. Briefly, cells (2×10³ in 50 µl/well) were seeded in 96-well plates. After 18 h of initial cell seeding, sodium selenite solutions were added, and the cells were incubated for 72 h. The cell proliferation rate and half maximal inhibitory (IC50) were then determined using a cell counting kit (Dojindo Lab., Tokyo, Japan) according to the manufacturer’s instructions. Absorbance was measured using a microplate reader (SpectraMax Plus 384, Molecular Devices, CA, USA).

**Clonogenic assay**

CHEK-1 cells were supplemented with various concentrations of sodium selenite (0-200 nM) for 72 h after 18 h of initial seeding and were then irradiated with 2 Gy X-ray irradiation. The cells (500 cells/4 ml medium) were seeded in 25cm² tissue culture flasks (Falcon, NJ, USA) immediately following irradiation. After 14 days of culture at 37°C, the cells were washed with PBS, fixed and stained with 0.5% crystal violet in H₂O:methanol (1:1) for 30 min at room temperature. Then, the cells were washed with tap water and air-dried. The total number of colonies with >50 cells was counted using a Binocular Light microscope (Olympus Corp). After counting the colonies, plating efficiency (PE) and survival fraction (SF) can be calculated using the following equations (22, 23):

\[
PE = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \times 100\%
\]

\[
SF = \frac{\text{Number of colonies formed post irradiation}}{\text{Number of cells seeded} \times \text{PE}} \times 100\%
\]

**Cell viability assay**

CHEK-1 cells (2×10³ in 50 µl/well) were seeded in 96-well plates. After 18 h of initial cell seeding, 50 nM sodium selenite solution was added to the cells, and they were incubated for 72 h and then irradiated. Post-irradiation cell viability was observed every 24 h for 72 h using a cell-counting kit solution (Dojindo Lab., Tokyo, Japan) according to the manufacturer’s instructions. The absorbance was measured using a microplate reader (SpectraMax Plus 384, Molecular Devices, CA, USA).
Cell cycle analysis
Both detached and attached cells were collected and then washed twice with ice-cold PBS at the end of each post-irradiation time point (24, 48 and 72 h). The cells were fixed with 70% cold ethanol and stored at -20°C until the day of analysis (1 to 4 days). On the day of analysis, cells were washed with PBS, stained with 0.05 mg/ml of propidium iodide (PI) solution (Sigma, St Louis, USA) with 0.002 mg/ml RNAse (Wako, Japan) and incubated at room temperature for 30 min. The DNA content was analyzed using the BD FACSCalibur™ (BD Biosciences, CA, USA).

Western blot analysis
Protein was extracted from the cells and measured after sodium selenite supplementation and 72 h post-irradiation. Protein (30 µg) samples were subjected to electrophoresis on a 5 to 20% SuperSepTM Ace ready gel (Wako, Japan) and electrotransferred to a nitrocellulose membrane (Amersham, Buckinghamshire, UK). The protein levels were analyzed using a poly ADP ribose polymerase (PARP) polyclonal antibody (Cell Signaling Technology #9542, MA, USA) at a 1:1000 dilution. An anti-rabbit IgG secondary antibody (NA934; Amersham, Buckinghamshire, UK) was used. Protein bands were detected using an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK). GAPDH (MAB374, Abcam) served as the loading control. Scanning densitometry was performed using Image Quant LAS 4000 (Amersham, Buckinghamshire, UK), and autoradiographs were quantified using the National Institute of Health’s ImageJ software program.

Statistical analysis
The differences between multiple variables were analyzed by one-way analysis of variance (ANOVA) and the Bonferroni pairwise comparison for the post-hoc analysis. A probability of p<0.05 was considered significant. All statistical analyses were performed by EZR statistical software program (24).

Results
Sodium selenite increased GPx-1 activity
In CHEK-1 cells, sodium selenite supplementation increased GPx-1 activity in a dose- and time-dependent manner (Figure 1). A previous study reported that 50 nM sodium selenite was used as a supplementation dose in primary keratinocytes prior to UV radiation treatment (25). In this study, when a 50 nM of sodium selenite solution was administered to CHEK-1 cells, the highest GPx-1 activity was achieved with a 72-h of incubation time. By examining multiple concentrations of sodium selenite with a 72-h incubation time, GPx-1 activity was found to be saturated at a concentration of 50 nM. The sodium selenite dose that resulted in the highest GPx-1 activity was 50 nM for 72 h, and these conditions were used for further experiments.
Figure 1. GPx-1 activity increased after sodium selenite supplementation in a dose- and time-dependent manner.
A. 50 nM sodium selenite supplementation affects GPx-1 activity in a time-dependent manner.
B. Sodium selenite supplementation for 72 h affects GPx-1 activity in a dose-dependent manner.
Results are from 3 independent experiments, and are presented as mean±SEM. *p<0.05 ** p < 0.01

Cytotoxicity of sodium selenite in CHEK-1 cells
Figure 2 depicts the cytotoxicity of sodium selenite in CHEK-1 cells based on the percentage of cell proliferation inhibition. The IC50 was 3.6 µM. The supplementation dose of 50 nM sodium selenite was assumed to be a low and safe dose for supplementation.

Figure 2. The IC50 of sodium selenite on CHEK-1 cells was 3.6 µM. Results are from 3 independent experiments, and are presented as mean±SEM.

Sodium selenite supplementation increased the post-irradiation cell survival rate
Post-irradiation survival of CHEK-1 cells was observed using a clonogenic assay and cell viability assay. Figure 3A presents the colony formation of the cells at 14 days post irradiation. Sodium selenite supplementation increased post-irradiation cell survival in a dose-dependent manner. Figure 3B depicts cell viability at 24 to 72 h post irradiation. At 72 h post-irradiation, the viability of the cells treated with 50 nM sodium selenite and 2 Gy irradiation was increased compare with 2 Gy irradiation alone. The results of both cell survival assays revealed that sodium selenite supplementation before irradiation protects cells from irradiation induced-damage post irradiation.

Figure 3. Post-irradiation CHEK-1 cell survival.
A. Colony formation assay using CHEK-1 cells. Sodium selenite supplementation increased the post-irradiation cell survival rate
B. Post-irradiation CHEK-1 cell viability. Sodium selenite supplementation increased cell viability at 72 h post-irradiation
Results are from 3 independent experiments, and are presented as mean±SEM. *p<0.05

Cell cycle
The cell cycle distribution of CHEK-1 cells post-irradiation is presented at Figure 4. The percentages of cells in the sub-G1, G1, G2/M, and S phases at 72 h in the untreated control group were 2.7±0.03, 71.2±5.01,
22.5±1.03, and 3.5±4.44, respectively, whereas the value in 50 nM sodium selenite group were 2.5±0.11, 64.6±3.21, 24.3±0.94, and 8.6±2.85, respectively. These results indicate that the cell cycle profile was not affected by treatment with sodium selenite with the exception of a moderate decrease in G1 cells from 71.2 to 64.6% between the control and sodium selenite groups. Treatment with 2 Gy X-ray irradiation resulted in an increase in the percentage of sub-G1 phase cells from 2.8 to 5.9% (p<0.05) and a decrease in the percentage of G1 phase cells from 71.2 to 58.8% at 72 h post-irradiation. Combined treatment with 50 nM sodium selenite and 2 Gy X-ray irradiation resulted in a reduced percentage of sub-G1 cells and an increased percentage of G1 cells at 72 h post-irradiation compared with 2 Gy X-ray irradiation alone [4.2% vs 5.9% (p<0.05), 62.1 vs 58.8%, respectively]. These results indicate that sodium selenite supplementation before irradiation reduces the percentage of apoptotic and damage cells and promotes entry into the G1 phase post-irradiation.

Figure 4. Cell cycle distribution of CHEK-1 cells post-irradiation
A. Percentage of cells in sub-G1, G1, G2/M, and S phases
B. Percentage of cells in sub-G1 phase of the cell cycle at 72 h after 2 Gy X-ray irradiation
Results are from 3 independent experiments, and are presented as mean±SEM. * p<0.05

Protein expression level of an apoptosis biomarker
The expression level of PARP protein, one of the best biomarkers of apoptosis, was analyzed 72 h post-irradiation. Figure 5 presents the expression of PARP and cleaved PARP proteins. GAPDH protein was used as a loading control. Treatment with 2 Gy X-ray irradiation increased the expression of cleaved PARP post-irradiation (p<0.05). In addition, the combination treatment of 50 nM sodium selenite and 2 Gy X-ray irradiation reduced the expression of cleaved PARP compared with 2 Gy X-ray irradiation alone. These results indicate that sodium selenite potentially inhibits radiation-induced apoptosis in noncancerous cells.

Figure 5. Protein expression level of an apoptotic biomarker
A. The protein expression levels of PARP and cleaved PARP
B. Relative density of the bands normalized to GAPDH
Results are from 3 independent experiments, and are presented as mean±SEM. * p<0.05

Discussion
Our study demonstrated that supplementation of noncancerous human esophageal cells with 50 nM sodium selenite before radiotherapy can protect the cells from radiation-induced damage and that this protection may be due to inhibition of radiation-induced apoptosis. These results agree with those of a
previous study (18) that used the organoselenium compound ebselen for supplementation on U937 cells prior to irradiation treatment.

Irradiation not only kills tumor cells, but also proliferating normal cells (2). Additionally, irradiation induces the production of ROS, which are causative agents of apoptotic cell death (26). One method of radioprotection involves inhibiting caspase activation and PARP cleavage (2). PARP cleavage is often associated with apoptosis and is a hallmark of apoptosis and caspase activation (27). By reducing the expression level of cleaved PARP, which is potential molecular target for new radioprotective compounds, we suggest that sodium selenite supplementation can serve as a radioprotective compound for normal cells when it is administered prior to clinical radiotherapy.

GPx is an important enzyme of cellular antioxidant defense systems that detoxifies peroxides and hydroperoxides. Selenocysteine is present at the catalytic site of GPx, and selenium availability regulates GPx enzyme activity (28). The stimulation of GPx activity following selenium supplementation indicates that the anti-oxidant function of this enzyme directly reduces the levels of oxidative DNA damage associated with radiation exposure (16). In this study we demonstrated that sodium selenite supplementation increases GPx-1 activity. This finding is in accordance with that of study by Diamond et al (16) indicating that low level supplementation of culture media with selenium in the form of sodium selenite significantly protected CHO-AA8 cells from radiation-induced mutagenesis and that this protection was associated with a significant elevation in GPx-1 activity.

This study investigated the protective effects of sodium selenite supplementation against irradiation-induced damage in noncancerous esophageal cells. Our results suggest that 50 nM sodium selenite supplementation for 72 h prior to irradiation protects normal cells from irradiation-induced damage by inhibiting irradiation-induced apoptosis; thus, sodium selenite is a prospective radioprotective compound for use in clinical radiotherapy. Husbeck et al (29) reported that the alteration of the redox environment of prostate cancer cells by sodium selenite supplementation increased their apoptotic potential and sensitized them to radiation-induced cell killing. However, the effects of sodium selenite supplementation on esophageal cancer cells remain unknown, and now we are currently investigating those effects.

Acknowledgement
This study was supported by a grant from GP 2013 (Gunma University, Japan).

References