Research Article

Sodium selenite supplementation does not reduce the effectiveness of X-ray irradiation treatment on human cancerous esophageal TE-8 cells

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Abstract

Sodium selenite supplementation at a concentration 50 nM before X-ray irradiation was suggested to protect non-cancerous human esophageal CHEK-1 cells from irradiation-induced damage. This present study investigated those effects on cancerous human esophageal cell line. The human cancer esophageal cell line, TE-8, was cultured and supplemented for the cytotoxicity assay, the GPx-1 activity, the cell viability assay, clonogenic assay and western blot analysis. An apoptosis biomarker, Cleaved PARP, was used. The results show that cell survival post-irradiation of supplemented-cells had the same effect as the cells treated by irradiation only, tended to decrease the cell viability (p=0.27), and decrease the survival rate of cancerous cells (p=1.00). The cleaved PARP level was higher in supplemented-and irradiated- cells than cells with irradiation alone. These results suggest that 50 nM sodium selenite supplementation prior to irradiation does not reduce the effectiveness of irradiation treatment on cancerous cells.

Keywords

Sodium selenite, X-ray, irradiation, supplementation, cancerous cell

Introduction

Radioprotective compounds are important in clinical radiotherapy, considering the significant damage radiotherapy can do to normal tissues and organs (Johnke et al. 2014). To prevent radiation damage in normal tissues, administration of radioprotective compounds has been recommended (Puspitasari et al. 2014). Selenium is one of the suggested radioprotective compounds for clinical radiotherapy (Puspitasari et al. 2014; Hosseinimehr 2015).

Selenium is an essential trace element and exists in many chemical forms in nature (Puspitasari et al. 2014).

In human systems, selenium integrated into various selenoproteins in the form of selenocysteine (SeCys). Several studies have provided evidence to show how selenium may benefit patients by mitigating the risk of several cancers (Rayman 2012). In addition, other studies have also reported that selenium can protect against the side effects induced by ionizing radiation (Hosseinimehr 2015).

Based on clinical studies conducted worldwide including European, American and Asian countries, between 1987 and 2012, selenium supplementation in the form of sodium selenite may offer benefits for cancer patients who undergo radiotherapy (Puspitasari et al.

2014). Selenium supplementation improved the general conditions and quality of life of patients and reduced the side effects of radiotherapy (Puspitasari et al. 2014). Taken orally at a dose of 200–500 µg/day, sodium selenite neither reduced the effectiveness of radiotherapy nor induced toxicities (Puspitasari et al. 2014).

Our previous study showed that 50 nM sodium selenite supplementation for 72 h was suggested to have the ability to protect non-cancerous human esophageal cells from a 2 Gy dose of X-ray irradiation in association with elevating the activity of glutathione peroxidase-1 (GPx-1) and reducing the cleaved PARP protein (Puspitasari et al. 2017). However, radioprotective compounds should ideally selectively protect normal tissues from radiation damage without protecting the cancer tissues (Citrin et al. 2010). Therefore, this present study investigated the effects of 50 nM sodium selenite supplementation on human esophageal cancer cells prior to X-ray irradiation treatment.

Methods

Cell culture

The human cancer esophageal cell line, TE-8 (Riken, Japan) (Hadisaputri et al. 2017) was cultured in RPMI-164 (Wako, Osaka, Japan) with 10% fetal bovine serum (Hy-Clone, Utah, USA) and 1% penicillin-streptomycin (Gibco, New York, USA) at 37 °C in a 5% CO_2 humidified chamber. To confirm that the cells were not in a selenium-deficient environment, we measured selenium concentration in the fetal bovine serum by using a modified Watkinson Method (Mutakin et al. 2016) on a Fluorometer (Twinkle LB970, Berthold Tech., Germany), and the concentration was 257.65±21.56 nM. By using 10% fetal bovine serum, the cells were grown in 25.76 ± 2.15 nM of selenium.

Selenium supplementation

Sodium selenite was purchased from Sigma (St. Louis, USA) and was supplemented at doses ranging from 0–16 µM for the assays of the cytotoxicity, 0–200 nM for the assays of the GPx activity, and 50 nM for the cell viability assay, clonogenic assay and western blot analysis. After 18 hours of initial seeding, the cells were incubated for 72 hours.

Irradiation

X-ray irradiation machine (Titan-225S, Shimadzu, Japan) was utilized to deliver irradiation treatment with a dose of 2 Gy at a rate of 1.3 Gy/min.

Cytotoxicity assay

The cytotoxicity of sodium selenite on the cells was examined with various concentrations of sodium selenite (0–16 µM) using a colorimetric assay and the half-maximal inhibitory value (IC50) were then determined using

cell counting kit-8 (Dojindo Lab., Tokyo, Japan) described in our previous study (Puspitasari et al. 2017). Briefly by adding 10 µl cell counting kit-8 solution to each well of the plate, incubating the plate for 2 hours in the incubator and measuring the absorbance at 450 nm using a microplate reader (SpectraMax Plus 384, Molecular Devices, CA, USA). The IC50 value was determined by linear regression analysis.

The value was estimated by using the equation in the form of y=ax+b, or, IC50=(50-b)/a (Puspitasari et al. 2017).

Cell viability

The cells ($2×10^3$ in 50 µl/well) were seeded in 96-well plates. After 18 hours of initial cell seeding, the cells were incubated for 72 hours in a 50 nM sodium selenite solution, and then irradiated. Following 72-hour post-irradiation, cell viability was observed using the cell- counting kit-8 solution (Dojindo Lab., Tokyo, Japan) according to the manufacturer's instructions. The absorbance was measured using a microplate reader (Puspitasari et al. 2017).

Clonogenic assay

The procedure for clonogenic assay has been described in our previous study (Puspitasari et al. 2017). Briefly, the cells were supplemented with various concentrations of sodium selenite (0- 200 nM), irradiated, then were immediately seeded in 25 cm^2 tissue culture flasks (Falcon, NJ, USA). The cells were cultured for 14 days at 37 °C. After washing with PBS, the cells were fixed with 99.5% ethanol and stained with 0.5% crystal violet in water:methanol (1:1). The colonies with >50 cells were counted using a Binocular Light microscope (Olympus Corp, Tokyo, Japan). After counting the colonies, the plating efficiency (PE) and survival fraction (SF) were calculated (Buch et al. 2012; Puspitasari et al. 2017).

Protein extraction

Extraction of the proteins from the cells was performed using RIPA buffer (Sigma, St. Louis, USA) with a 10% protein inhibitor (Sigma, St. Louis, USA) (Puspitasari et al. 2017). The protein concentrations were then determined using a Bio-Rad DC protein assay kit (Bio-Rad, Tokyo, Japan).

GPx-1 activity assay

The enzymatic activity of GPx-1 in TE-8 cell homogenates was determined using the method described by Paglia and Valentine at a time- and dose-dependent manner, with certain modifications that have been described in our previous study (Puspitasari et al. 2017). Briefly, GPx-1 activity was indirectly monitored spectrophotometrically by the reduction of oxidized glutathione using nicotinamide adenine dinucleotide phosphate (NADPH) as the reducing agent at 340 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Western blot analysis

Cell protein was extracted and measured after irradiation of the cells supplemented with sodium selenite for 72 hours. The Western blot analysis method has been described in our previous study (Puspitasari et al. 2017). Briefly, 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 and a cleaved PARP (Asp214) antibody (Human Specific) (Cell Signaling Technology #9541, MA, USA) at a 1:1000 dilution. An anti- rabbit IgG secondary antibody (NA934; Amersham, Buckinghamshire, UK) was used to detect the antibodies.

Statistical analysis

All experiments were repeated three times, and the results are presented as the mean ± standard error. 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 in all tests. All statistical analyses were performed by EZR statistical software program, an open-source statistical software program based on R and R commander version 3.3.1 (Kanda 2013).

Results

The IC50 of sodium selenite in cancerous esophageal TE-8 cells

Fig. 1 presents the cell proliferation and cytotoxity of sodium selenite supplementation on cancerous (TE-8) esophageal cells. The IC50 of sodium selenite in human cancerous TE-8 cells was 7.23 μM. Based on previous results (Puspitasari et al. 2017), this result showed that the IC50 of cancerous cells was higher than non-cancerous (3.6 μM) cells toward sodium selenite. It revealed that non-cancerous cells are more sensitive to sodium selenite than cancerous cells.
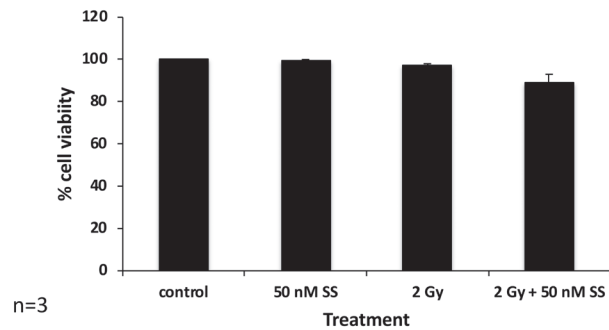
Cells survival post-irradiation

Cell survival of cancerous and non-cancerous cells was assessed post-irradiation with the cell viability and clonogenic assays. Fig. 2 shows the cell survival of cancerous esophageal cells post-irradiation. If the control group in the clonogenic assay was compared with 2 Gy irradiation group and to 2 Gy + 50 nM sodium selenite group, the differences for both comparisons were significant (p = 0.00). In addition, sodium selenite-supplemented TE-8 cells treated with irradiation were less viable than the cells treated only with irradiation although the difference was not
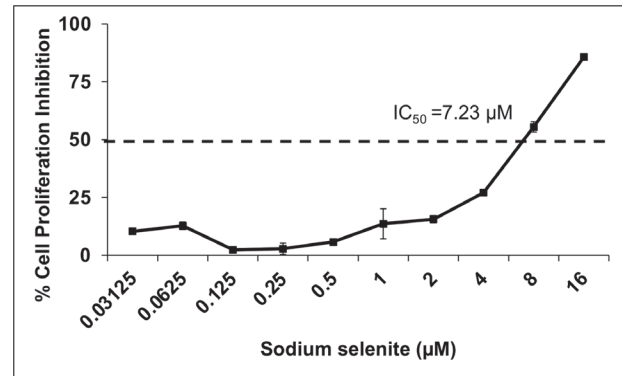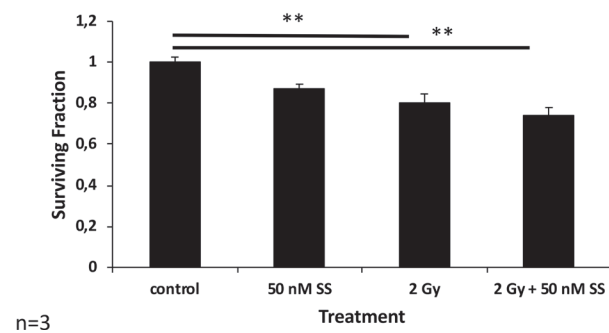

Figure 1. The cell cytotoxicity of sodium selenite supplementation on human cancerous esophageal TE-8 cells. The half-maximal inhibitory concentration (IC50) of sodium selenite on TE-8 cells was 7.23 μM. n=number of samples analyzed.

2A.

2B.

Figure 2. Cells survival post-irradiation. **2A.** Cell viability result showed that sodium selenite supplementation prior to irradiation tended to decrease the cell viability. SS=Sodium Selenite; **2B.** Clonogenic assay showed that sodium selenite supplementation prior to irradiation tended to decrease the survival rate of human cancerous TE-8 cells. ** p<0.01 compared to control. n=number of samples analyzed. SS=Sodium Selenite.

significant (p = 0.27) and the results of the clonogenic assay showed the same trends as the cell viability assay (p = 1.00). That result indicated that sodium selenite supplementation prior to irradiation has the same effectiveness as the cells treated by irradiation only. This signifies that sodium selenite supplementation sensitizes radiation-induced cell- killing but does not reduce the effectiveness of radiation treatment on cancerous cells.
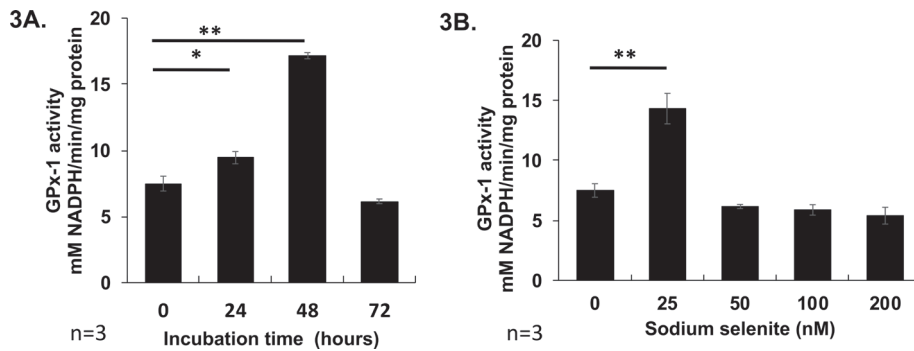
Figure 3. GPx-1 activity of TE-8 cells in a time- and dose-dependent manner. **3A.** GPx-1 activity of TE-8 cells in a time dependent manner with a 72-hour incubation time was determined to be 6.15 ± 0.17 mM NADPH/min/mg protein. ** p<0.01, *p<0.05; **3B.** GPx-1 activity of TE-8 cells in dose dependent manner was highest at dose 25 nM of sodium selenite supplementation. ** p<0.01.

## GPx-1 activity of the cells supplemented with sodium selenite

GPx-1 activity of cancerous TE-8 cells was observed in a time- and dose-dependent manner, as shown in Fig. 3. The activity of cancerous TE-8 cells with a 72-hour incubation time was determined to be 6.15 ± 0.17 mM NADPH/min/mg protein, while the activity on non-cancerous CHEK-1 cells in previous study was 40.26 ± 4.09 mM NADPH/min/mg protein. The highest activity of GPx-1 in supplemented with 50 nM sodium selenite cancerous TE-8 cells was obtained in the 48-h incubation time (17.15 ± 0.23 mM NADPH/min/mg protein) and the highest GPx-1 activity of TE-8 cells was obtained at a dose 25 nM at 72 h incubation time (14.31 ± 1.27 mM NADPH/min/mg protein).

Western blot with apoptosis biomarkers post-irradiation

The cells proteins were analyzed by Western blot analysis 72-hour post-irradiation with apoptosis biomarkers PARP and cleaved PARP (Fig. 3). The cleaved PARP level treated with sodium selenite and then irradiated was higher (relative density = 1.69) than the cells treated with irradiation alone (relative density = 1.61). Sodium selenite supplementation tended to elevate the cleaved PARP level in irradiated cancerous cells although the difference was not significant in value (p = 1.00).

Discussion

The present study demonstrated that 50 nM sodium selenite supplementation in cancerous TE-8 cells prior to 2 Gy X-ray irradiation has the opposite effect with our previous study on non-cancerous cells. In our previous study, with non-cancerous cells, 50 nM sodium selenite supplementation has a protective effect against X-ray irradiation, meanwhile, in present study it showed that 50 nM sodium selenite supplementation on human cancerous TE- 8 cells did not protect the cells from X-ray irradiation by decreasing
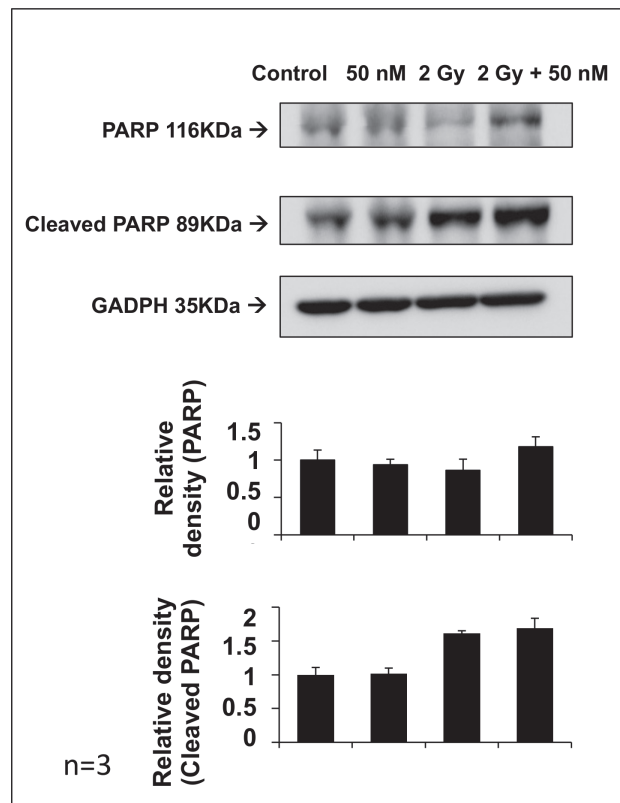
Figure 4. Western blot analysis results with apoptosis biomarkers. Cleaved PARP was higher in human cancerous TE-8 cells with sodium selenite supplementation and irradiation (relative density=1.69) than the cells treated with irradiation alone (relative density=1.61). n=number of samples analyzed.

cell viability and survival rate post-irradiation and did not reduce the effectiveness of irradiation treatment.

In other previous in-vitro selenium supplementation with irradiation studies, they used lower dose sodium selenite supplementation (De Rosa et al. 2012), lower dose irradiation (Eckers et al. 2013), and also UV irradiation for treatment (De Rosa et al. 2012). However, our study used 50 nM of sodium selenite supplementation based on the highest GPx-1 activity in our previous study that can protect non-cancerous human esophageal

CHEK-1 cells from irradiation treatment and used 2 Gy X-ray irradiation based on common fractionation dose for radiotherapy at clinical cancer treatment (Puspitasari et al. 2017).

GPx was assumed to be associated with the antioxidant activity of selenium (Reinke et al. 2014). GPx1 is an intracellular antioxidant enzyme that may modulate overall redox stress by reducing reactive oxygen species (ROS)(Meng et al. 2018). Decreased GPx-1 activity can increase sensitivity to oxidative stress leading to accumulation of harmful oxidants, conversely, excess GPx-1 can increase reductive stress, which is characterized by a lack of essential ROS required for cellular signalling processes (Lubos et al. 2011). Lack of essential ROS can lead to reduced cell growth and promote apoptotic pathways (Lubos et al. 2011). In the present study, the activity of GPx-1 on a 72 h 50 nM selenium supplemented cancerous TE-8 cells was much lower (6.15 ± 0.17 mM NADPH/min/mg protein) than the activity on the non-cancerous CHEK-1 cells from previous our study (40.26 ± 4.09 mM NADPH/min/mg protein) (Puspitasari et al. 2017). The highest activity of GPx-1 in cancerous TE-8 cells supplemented with 50 nM sodium selenite was obtained in the 48-h incubation time (17.15 ± 0.23 mM NADPH/min/mg protein). Therefore, at 72 h the activity had been decrease to plateau and it was suggested did not protect the cells from irradiation. On the other hand, the highest GPx-1 activity of TE-8 cells was obtained at a dose of 25 nM at 72 h incubation time (14.31 ± 1.27 mM NADPH/min/mg protein). A study confirmed that sodium selenite supplementation at a dose of 30 nM for 72 h incubation time on human cancerous prostate LNCaP cells increased oxidative DNA repair activity which can protect the cells from oxidative stress (De Rosa et al. 2012). It indicated that supplementation dose selection is important to be concerned, since sodium selenite supplementation was suggested has the ability to protect non-cancerous cells (Puspitasari et al. 2017) and radiosensitize cancerous cells (Calvaruso et al. 2019).

PARP-1 is one of the cellular substrates of caspases that once activated could initiate cell death (Chaitanya et al. 2010). Cleavage of PARP-1 by caspases is considered to be a biomarker of apoptosis (Chaitanya et al. 2010). Blocking PARP cleavage is one of the possible mechanisms proposed for developing future radiation protector in a review paper (Greenberger 2009). Reversing the possible mechanism of radiation protector could result in the development of radiosensitizers or agents that increase cellular capacity to respond to ionizing radiation (Greenberger 2009). In our previous study, 50 nM sodium selenite supplementation for 72 h was suggested to have the ability to protect non-cancerous human esophageal cells from a 2 Gy dose of X-ray irradiation by increasing survival rate and reducing the cleaved PARP protein, in contrast, in this study, 50 nM sodium selenite supplementation on the cells prior to X-ray irradiation tended to decrease the survival rate of cancerous cells with the same effectiveness as the cell with irradiation alone (p = 0.00) and tended to elevate the cleaved PARP level in irradiated cancerous cells although the difference was not significant in value (p = 1.00). Decreasing survival rate and increasing the cleaved PARP level indicated that more cancerous cells undergo apoptosis and leading to cell death so that the supplementation did not reduce the effectiveness of X-ray irradiation treatment.

It is important to mention that by adding 10% fetal bovine serum on culture media, the cells in our study were grown in 25.76 ± 2.15 nM of selenium, with regards to the selenium- deficient environment. In another study, the selenium level from the serum was measured and was about 248.25 ± 45 nM by using 10% serum (De Rosa et al. 2012). The cells in that study were grown in 24.825 ± 4.5 nM.

The limitation of this study was only conducted in one cancerous TE-8 cell line and only providing limited apoptosis biomarkers (PARP and cleaved PARP) as a molecular target for possible mechanisms. In addition, further experiments are needed to explore the radiosensitizing effect of sodium selenite on cancerous cells. However, our study could indicate that supplementation sodium selenite in TE-8 cancerous cells prior to irradiation treatment does not protect the cells against irradiation and does not reduce the effectiveness of X-ray irradiation treatment.

Conclusion

A low-dose sodium selenite supplementation, 50 nM for 72 hours prior to X-ray irradiation does not reduce the effectiveness of X-ray irradiation treatment. Further experiments with more cell lines, more careful dose selection and investigating other molecular targets for possible mechanisms should be conducted to confirm the findings of the current study.

Declaration of interest statement

The authors report no conflict of interest.

Authors contributions

IMP was responsible for the study design. IMP and RA were responsible for data collections and analysis. All authors participated in the drafting and revising of the manuscript. All authors read and approved the final manuscript.

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