Sodium selenite inhibits the proliferate of cervical cancer cells through PI3K/AKT pathway

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Abstract

Selenium can inhibit cervical cancers, but the specific mechanism of anti-cervical cancer is not fully understood. In this study, we investigated the effect of sodium selenite (SS) on cervical cancer cell lines HeLa and SiHa and examined the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway to reveal the mechanism of SS against cervical cancer. We detected cell viability by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide] assay, and apoptosis by Annexin V-FITC/PI staining. The levels of PI3K, AKT, phosphorylated PI3K (p-PI3K), and phosphorylated Akt (p-AKT) were measured by Western Blot. The results showed that SS reduced the viability and increased apoptosis of HeLa and SiHa cells and suppressed the activation of the PI3K/AKT signaling pathway in a dose-dependent manner. To verify the role of PI3K/AKT signaling pathway in this, we treated HeLa and SiHa cells with LY294002 (LY, a specific PI3K inhibitor) and established control, LY-treated, SS-treated, and combined LY + SS-treated groups. The results showed that the combined LY + SS treatment group enhanced the inhibitory effect of SS on the PI3K/AKT signaling pathway, which further inhibiting cervical cancer cell viability and increasing apoptosis. In conclusion, SS exerted its anti-cervical cancer effects by inhibiting cell proliferation, promoting apoptosis, and inhibiting PI3K/AKT signaling pathway.

Introduction

Cervical cancer is the fourth most common cause of cancer-related deaths among women worldwide, after breast, colorectal, and lung cancers [1]. In 2020, there were approximately 604,127 new cases of cervical cancer and 341,831 new deaths from cervical cancer worldwide [1]. Surgery followed by chemotherapy can cure 80–90% of women with early-stage cervical cancer, but the ineffectiveness of conventional treatment for advanced or recurrent cervical cancer remains an unresolved problem [2, 3]. Therefore, new drugs are still needed for the treatment of cervical cancer.

Selenium is an essential trace element with significant physiological functions in human health [4]. Selenium deficiency may increase the risk of cervical cancer [5] and breast cancer [6]. Studies reported that selenium supplementation inhibits the proliferation of tumors such as thyroid cancer [7], lung cancer [8], and colon cancer [9]. Clinical studies have found that low selenium may affect the effectiveness of treatment for cervical cancer [10], and selenium supplementation can improve the treatment of patients with gynecological tumors [11, 12]. Muecke et al found that oral supplementation with 500 µg/d of sodium selenite (SS) during radiotherapy in selenium-deficient patients with cervical and uterine cancer increased blood selenium concentrations and reduced the number and severity of radiotherapy-induced diarrhea [13]. These studies suggest that selenium may be used as a potential anti-cancer molecule and for future clinical applications. Selenium inhibits tumor growth and development through its anti-proliferation, immunomodulatory, anti-inflammatory, pro-apoptotic, and anti-migration effects [14]. Mechanism studies showed that the anti-cancer effects of selenium might be related to the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway [15]. PI3K/AKT signaling pathway is a classical intracellular pathway that plays an essential role in apoptosis and tumor development [16]. Although studies have confirmed that the PI3K/AKT signaling pathway plays a crucial
role in the occurrence, development, and progression of cancer [17–19], no studies have confirmed its role in regulating cervical cancer by SS. Whether SS inhibits the growth of cervical cancer by regulating PI3K/AKT signaling pathway remains to be studied.

**Materials and Methods**

**Chemistry**

In this study, SS was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Selenite powder was dissolved in sterile water to prepare a 10 mmol/L stock solution of selenite and stored at -20°C for a final concentration of 5 or 7.5 µmol/L. PI3K inhibitor LY294002 (LY) was purchased from Absin (Shanghai, China). A stock solution of 20 mmol/L was prepared by dissolving LY powder in dimethyl sulfoxide (DMSO) and stored at -20°C for a final concentration of 20 µmol/L. For inhibitor studies, 20 µM LY was pre-treated to the culture solution before the SS solution for one hour.

**Cell culture**

Human cervical cancer cell lines [HeLa (human papilloma virus 18 type cervical cancer cell line) and SiHa (human papilloma virus 16 type cervical cancer cell line)] were purchased from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). The HeLa and SiHa cells were cultured in complete RPMI-1640 and DMEM medium (supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic agents) respectively, at 37°C humidified 5% CO₂ incubator. Cells were passaged more than 3 times for experiments. All cell lines we used in this study were mycoplasma free.

**Cell viability assay**

Cell viability (survival) was determined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Human cervical cancer cell lines (HeLa and SiHa) were seeded in 96-well plates at a density of 10⁴ cells per well and allowed to grow for 24 h. The cells were treated with SS in the presence or absence of LY for 24 h. Then we added 5 mg/mL MTT solution (1334GR001, Biofroxx, Guangzhou, China) at 37°C for 4 h, discarded the supernatant, added 150 µL DMSO to each well and shook it for 10 min in the dark to fully dissolve the formazan. The absorbance was measured at 490 nm wavelength using a BioTek Cytation 3 (BioTek, Instruments, USA). The half-maximal (50%) inhibitory concentration (IC₅₀) value of SS in each cell line was calculated. IC₅₀ values reflect 50% inhibition of cell viability.

**Cell morphology**

To examine the morphological changes with the effect of the drug, both HeLa and SiHa cells were seeded in 6-well plates and incubated overnight. Subsequently, the cells were treated with desired concentrations
of SS for 24 h. Afterwards, the cells were visualized under the phase-contrast microscope at 100 × magnification (Olympus, Tokyo, Japan).

**Apoptosis**

Cells in the medium and adherent cells were all collected and stained using an Annexin V- fluorescein isothiocyanate (FITC)/PI Apoptosis Detection Kit (Absin, Shanghai, China). After collection by centrifugation and washing with ice-cold PBS, cells were re-suspended in 300 µL of binding buffer. Then cells were incubated with Annexin V-FITC and PI dye under room temperature in the dark for 15 min and 5 min, respectively, and examined by flow cytometer (AccuriTM C6, BD Biosciences, USA). The apoptotic rate (%) was calculated as the sum of Annexin V-FITC+/PI- (early apoptosis, Q3) and Annexin-V-FITC+/PI+ (late apoptosis, Q2) cells.

**Western blotting**

Whole cell lysates were prepared from cell lines by RIPA buffer (Beyotime, Shanghai, China). The protein concentrations were quantified using a BCA protein assay kit (Beyotime, Shanghai, China). The quantified protein was adjusted to the same concentration, then a 4×loading buffer was added and mixed, and the protein was denatured at 95 °C for 5 min. 20–30 µg of proteins per sample was separated by 10% SDS-PAGE, then transferred onto PVDF membranes, and blocked with 5% skimmed milk for 1 h at room temperature. The membrane was incubated with primary antibodies, specific to PI3K (Cell Signaling Technology, # 4257, 1:1000), p-PI3K (Tyr 607, Affinity, #AF3241, 1:1000), AKT (Cell Signaling Technology, #9272, 1:1000), p-AKT (Ser 473, Cell Signaling Technology, #4060, 1:2000), and β-actin (ZSGB-BIO, TA-09, 1:2000) at 4 °C overnight. The following day, after washing three times with TBST, a goat anti-rabbit (1:5000) or goat anti-mouse (1:5000) secondary antibodies were added for incubation for 1 h at room temperature and washed three times with TBST. Finally, the protein bands were imaged using an enhanced chemiluminescent substrate, the images were captured on the visualization instrument Tanon-5200 (Tanon, China). The results were expressed as a relative optical density and analyzed using ImageJ software. Values based on three independent experiments were used for statistical analysis.

**Statistical analysis**

Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as the means ± standard deviation (means ± SD) with significance p-values of < 0.05. Student’s t-test was used to determine differences between two groups, and one-way analysis of variance (ANOVA) was used to determine the differences between three or more groups. The post hoc analysis was carried out to compare the significance between groups by Dunnett T3 test or LSD test. If the variances of the data were not equal, the nonparametric Kruskal Wallis test was used for statistical evaluations. Statistical significance is described in the figure legends as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Results

SS reduced the viability of cervical cancer HeLa and SiHa cells

To investigate the effect of SS on the viability of cervical cancer HeLa and SiHa cells, we performed an MTT assay. As shown in Fig. 1a, SS significantly reduced the cell viability of HeLa and SiHa cells in a dose-dependent manner. The 24 h IC$_{50}$ of HeLa and SiHa cells were 6.508 µM and 8.825 µM, respectively (Fig. 1b). As shown in Fig. 1c, the SS-treated group caused a series of morphological changes, such as cell shrinkage, irregular cell shape, and increased suspended cells, compared with the control group.

SS promoted apoptosis in HeLa and SiHa cells

To investigate whether SS induces apoptosis, we detected apoptosis by flow cytometry using Annexin V-FITC/PI double-staining assay. As shown in Fig. 2, in both HeLa and SiHa cell lines, the percentage of apoptosis increased compared to the control group in a dose-dependent manner. In HeLa cell lines, the percentage of apoptosis increased from 4.44 ± 0.24% in the control group to 5.5 ± 0.56% in the 2.5 µM SS-treated group and 8.88 ± 0.2% in the 5 µM SS-treated group, respectively. In SiHa cells, the percentage of apoptotic cells increased from 9.8 ± 0.18% in the control group to 21.29 ± 0.54% in the 5 µM SS-treated group to 25.88 ± 0.85% in the 7.5 µM SS-treated group, respectively.

SS dose-dependently suppressed the activation of PI3K/AKT signaling pathway

We examined the expression of PI3K/AKT pathway proteins after SS treatment. In SS-treated HeLa and SiHa cells, we found no significant difference in the total protein levels of PI3K and AKT, while the phosphorylation modification levels of the Thy607 site of PI3K protein and the Ser473 site of AKT protein gradually decreased with increasing SS dose, as shown in Fig. 3.

LY enhanced the effect of SS inhibition of PI3K/AKT signaling pathway

To investigate the role of PI3K/AKT signaling pathway in SS inhibition of cervical cancer cell proliferation and promotion of apoptosis, we first evaluated the effect of LY294002 (LY, a specific PI3K inhibitor). As shown in Fig. 4, after 20 µM LY pretreatment for 1 h, a decrease in p-PI3K and p-AKT protein levels was observed in both HeLa and SiHa cell lines in the LY pretreatment group alone, and the inhibitory effect was slightly lower than that in the SS treatment group alone, while LY pretreatment combined with SS intervention for 24h could significantly enhance the inhibitory effect of SS on p-PI3K and p-AKT.

LY significantly enhanced the inhibitory effect of SS on the viability of HeLa and SiHa cells
Further, we investigated the effects of LY and SS on the proliferation of cervical cancer cells. Compared with the control group, cell viability decreased in both the LY-treated and SS-treated groups, and significantly decreased in the combined LY + SS-treated group (P < 0.0001). As shown in Fig. 5a, cell viability was significantly reduced in the 5 µM SS-treated group for HeLa cells and 7.5 µM SS-treated group for SiHa cells compared with the control group, down to 69.55% and 66.46%, respectively. Cell viability was reduced but to a lesser extent in the LY-treated group, down to 92.70% in HeLa and 91.84% in SiHa. The combined LY + SS-treated group further reduced the viability of both HeLa and SiHa cells to 40.01% and 41.00%, respectively. We also observed the same results when observing HeLa and SiHa cell morphology by microscopy. As shown in Fig. 5b, compared with the control group, the LY pretreatment group showed no significant changes in cell morphology and the number of suspended cells, the SS-treated group showed irregular changes in cell morphology, morphological crinkling, and an increase in the number of suspended cells, while the combined LY + SS treatment group showed more significant phenomena such as an increase in suspended HeLa and SiHa cells and a decrease in live cells.

**LY enhanced the pro-apoptotic effect of SS on HeLa and SiHa cells**

To elucidate whether the PI3K/AKT pathway has a key role in SS-induced apoptosis in cervical cancer cells, this study examined the effect of LY on SS-induced apoptosis in HeLa and SiHa by the flow cytometry using Annexin V-FITC/PI method. The apoptosis results showed that compared with the control group, apoptosis of HeLa and SiHa cells was significantly increased in both the LY-treated and SS-treated groups. The degree of apoptosis in the combined LY + SS-treated group was further deepened compared with the SS-treated group, and the percentage of apoptosis in HeLa cells increased from 9.06 ± 0.18% in the SS-treated group to 21.42 ± 1.13% in the combined group, and in SiHa cells increased from 21.19 ± 0.99% to 54.87 ± 0.53% (Fig. 6).

**Discussion**

Selenium is essential trace elements that play an essential role in the physiological processes and functions of human health [4]. Studies have shown that selenium has anti-tumor effects [20]. SS can induce apoptosis in pancreatic cancer [2] (PANC-1 and Pan02 cells), human glioblastoma[21] (R2J-GS cells), breast cancer[22, 23] (MCF7, 4T1 cells), and other tumor cells. SS inhibited the proliferation of cervical cancer cells, but its exact mechanism has not been fully elucidated. Therefore, we explored the possible mechanisms of SS anti-cervical cancer.

Cell viability assays and morphological observations showed that SS decreased the viability of HeLa and SiHa cells, resulted in irregular cell morphology, increased the number of suspended cells, and decreased the number of viable cells. The above results indicated that SS inhibited HeLa and SiHa cell growth, which suggested that SS has anti-cervical cancer effects. Our study found that SS induced apoptosis in HeLa and SiHa cells. We further found SS induced apoptosis in cervical cancer cells, probably by inhibiting the PI3K/AKT signaling pathway.
In the present study, we examined the expression of the PI3K/AKT signaling pathway proteins after SS intervention in cervical cancer cells and showed that SS significantly inhibited the level of p-PI3K and p-AKT proteins. These findings suggested that SS may promote apoptosis in cervical cancer cells by inhibiting the PI3K/AKT pathway. To further clarify the role of the PI3K/AKT pathway in the inhibition of HeLa and SiHa cell viability and induction of apoptosis by SS, we inhibited the PI3K/AKT pathway with the inhibitor of this pathway, LY. The results revealed that LY pretreatment combined with SS significantly enhanced the inhibitory effect of SS on p-PI3K and p-Akt protein levels, cell proliferation inhibition, and induction of apoptosis. The possible explanation is that LY inhibited the phosphorylation of PI3K, which led to the inhibition of phosphorylation activation of the downstream effector molecule AKT, which then affected the SS-induced changes in cervical cancer cell viability and apoptosis.

Studies have shown that signaling in the PI3K/AKT pathway relies primarily on changes in the phosphorylation levels of crucial upstream molecules to activate downstream protein factors[24]. Activated AKT, as a key molecule in the PI3K/AKT signaling pathway, can induce a series of downstream phosphorylation cascades and participate in multiple processes such as cell cycle, proliferation, and apoptosis [25]. It was shown that SS inhibited cervical cancer cell proliferation and induced apoptosis by inhibiting the PI3K/AKT signaling pathway. Other studies have reported a similar view that SS induces thyroid cancer cell cycle arrest and apoptosis through reactive oxygen species-dependent inhibition of the AKT/mTOR pathway [7]. Arctiin inhibits migration and invasion of cervical cancer cells by suppressing S100A4 expression through the PI3K/AKT pathway [26].

The present study demonstrated for the first time that SS inhibited proliferation and induced apoptosis in cervical cancer cells by inhibiting the PI3K/AKT signaling pathway. The results suggested that PI3K/AKT signaling pathway plays an essential role in SS against cervical cancer cells. However, it still needs to be further validated by activation experiments, although we have chosen an inhibitor for the experiments which is closer to the treatment of cervical cancer. In addition, the lack of in vivo experiments is a limitation of this study. In future studies, we will explore the effects of SS on cervical cancer cells in vivo to provide more evidence for the mechanism by which SS inhibits cervical cancer cell proliferation.

Conclusion

SS dose-dependently inhibited the proliferation of cervical cancer HeLa and SiHa cells. The mechanism of its anticancer effect may be caused by inhibiting the PI3K/AKT signaling pathway and promoting cell apoptosis.

Declarations

**Competing Interests:** The authors declare no competing interests.

**Ethics approval:** This paper does not contain any studies with human or animals.

**Data Availability:** Not applicable.
Consent to participate: Not applicable.

Consent to publish: Not applicable.

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Author Statement

YW, LQ, TW, and QL contributed to conception and design of the study. YW, LQ, SH, CF, and LD performed the experiments. YJ, RW, GL, and JL performed the statistical analysis. MW, SS, HS, CL, and QZ were responsible for data analysis and visualization. YW wrote the first draft of the manuscript. QL and TW reviewed and edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

References


Figures
Figure 1

SS reduced the viability of cervical cancer HeLa and SiHa cells. (a) Cervical cancer cell lines were exposed to different concentrations of SS for 24 h and the MTT assay was performed to evaluate cell viabilities. (b) IC50 values were calculated by nonlinear regression analysis on a dose-response curve. (c) Cell morphology was observed by phase-contrast microscopy (magnification, ×100).
Figure 2

SS enhanced apoptosis in HeLa and SiHa cells. (a-b) Apoptosis analysis was performed by flow cytometry after 24 h treatment with 2.5, 5 μM and 5, 7.5 μM SS in HeLa and SiHa, respectively. Cells were stained with Annexin V-FITC to detect apoptotic cells and PI for nuclear stain. Results were considered significant vs. control when *p < 0.05.

Figure 3

SS dose-dependently reduced PI3K/AKT signaling pathway protein levels. (a-d) The expression of PI3K, phosphorylated PI3K (Try607), AKT, and phosphorylated AKT (Ser 473) were detected by Western blotting. The expression of β-actin was used as a reference. Statistical differences vs. control were expressed with superscript symbols: * p< 0.05, ** p < 0.01, *** p < 0.001, respectively.
**Figure 4**

LY significantly enhanced the inhibitory effect of SS on PI3K/AKT signaling pathway. (a-d) HeLa and SiHa cells were pre-treated with or without 20 μM LY for 1 h, followed by 5 μM and 7.5 μM SS for 24 h, respectively. LY pre-treatment further reduced the protein levels of p-PI3K and p-AKT reduced by SS treatment in HeLa and SiHa cells. The expression of β-actin was used as a reference. Statistical differences vs. control were expressed with superscript symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and vs SS+LY were expressed with superscript symbols # $p < 0.05$, ## $p < 0.01$, respectively.
Figure 5

LY significantly enhanced the inhibitory effect of SS on the viability of HeLa and SiHa cells. HeLa and SiHa cells were pre-treated with or without LY for 1 h, followed by 5 μM and 7.5 μM SS for 24 h, respectively. (a) Cell viability was measured by MTT assay. (b) Cell morphology was observed by phase-contrast microscopy (magnification, ×100). Statistical differences vs. control were expressed with superscript symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and vs SS+LY were expressed with superscript symbols # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$, respectively.
Figure 6

LY significantly enhanced the effect of SS on increasing apoptosis in HeLa and SiHa cells. (a-b) Cells were pre-treated with or without LY for 1 h followed by 5 or 7.5 μM SS treatment for 24 h. Apoptosis analysis was evaluated by flow cytometry. Statistical differences vs. control were expressed with superscript symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and vs SS+LY were expressed with superscript symbols # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$, respectively.