Abstract. This study investigated the effect of sodium selenite (Na$_2$SeO$_3$) on proliferation, cell cycle, apoptosis as well as the underlying mechanism in CNE-2 nasopharyngeal carcinoma (NPC) cells. The CNE-2 cell line was treated with different concentrations of Na$_2$SeO$_3$, and the effects of Na$_2$SeO$_3$ on cell viability and proliferation were evaluated using Cell Counting kit-8 (CCK-8) assay. Cellular apoptosis and cell cycle were evaluated by flow cytometry following Annexin V-FITC/PI double staining and PI single staining respectively; nuclei morphology stained with DAPI and Hoechst 333258 was observed under a fluorescence microscope, while DNA fragmentation was detected by agarose gel electrophoresis. The mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) were analyzed using fluorescent staining assays. Expression of Bcl-XL, Bax, Bak, and caspase-3 activation were examined by western blotting. The results showed that Na$_2$SeO$_3$ inhibited proliferation and induced apoptosis of CNE-2 cells in a dose- and time-dependent manner. Na$_2$SeO$_3$ at low concentrations induced cell cycle arrest at S phase, while high concentrations of Na$_2$SeO$_3$ induced cell cycle arrest at G$_0$/G$_1$ phase. Furthermore, Na$_2$SeO$_3$ increased ROS level and decreased MMP, upregulated caspase-3 activity and the expression of Bak and Bax but simultaneously downregulated Bcl-XL. In conclusion, our studies demonstrated that Na$_2$SeO$_3$ had significant anti-proliferative and apoptosis-inducing effects via arresting cell cycle and regulating mitochondria-mediated intrinsic caspase pathway in CNE-2 NPC cells, suggesting that Na$_2$SeO$_3$ might have therapeutic potentials in the treatment of NPC.

Introduction

Apoptotic cells are morphologically characterized by membrane blebbing, chromatin condensation, and the formation of apoptotic bodies. Apoptosis is a cell suicide mechanism that is regulated by two canonical programmed cellular signaling pathways; the death receptor-mediated pathway (extrinsic) and the mitochondrial pathway (intrinsic). Activation of caspases is important in both pathways (1,2). Interaction between ligands and death receptors initiates the extrinsic pathway at the plasma membrane, subsequently activating caspase-8, which is an initiator caspase. This protein, in turn, directly activates downstream effector caspases, including caspase-3 (3). Many physical and chemical stimuli induce mitochondrial dysfunction and changes in reactive oxygen species (ROS) production, triggering the intrinsic pathway. Mitochondrial dysfunction induces the activation of caspase-9 and subsequently activates effector caspases, such as caspase-3. Following the activation of caspase-3, several specific substrates are cleaved (4,5). Apoptosis is also associated with ROS, mitochondrial membrane potential (MMP) and other relevant factors. Apoptosis induction is a critical mechanism for numerous anti-cancer compounds (6).

Selenium (Se) is an essential trace element (7), and appropriate Se intake is necessary for the body to synthesize selenoproteins. Several studies have indicated that sodium selenite (Na$_2$SeO$_3$) inhibits growth of a series of cancer cell lines, including liver and prostate cancer, malignant melanoma and various hematologic malignancies, by inducing apoptosis via different mechanisms including mitochondria, oxidative stress, p53-dependent signaling, and thioredoxin reductase (8-11). Na$_2$SeO$_3$ in particular exerts antitumor effects by inducing apoptosis (12-17).

Nasopharyngeal carcinoma (NPC) is the most common epithelial malignancy of the nasopharynx. The pathogenesis of NPC is yet not clear, and effective, low-toxicity therapies are not available so far. Thus, research and development of potential drug candidates for NPC are of utmost importance. In this study, we investigated the anti-cancer effects and mechanisms...
of Na₂SeO₃ in CNE-2 NPC cells. We found that Na₂SeO₃ can inhibit cell proliferation and induce cell apoptosis via cell cycle arresting and mitochondrial pathways.

Materials and methods

Materials. Na₂SeO₃ was purchased from Food and Drug Administration of China (Beijing, China) (lot no. 110713-200911) and dissolved in Milli-Q water to get a stock concentration of 1 mM, then stored at -20°C until use. The Cell Counting kit-8 (CCK-8) (cat. C0038), MMP assay kit with 5,5',6,6'-tetrachloro-1',3,3'-tetraethylbenzimidazolyl carboxcyanine iodide (JC-1) (cat. C2006), ROS assay kit (cat. S0033), DNA Ladder assay kit (cat. C0007), DAPI staining kit (cat. C1005) and Hoechst 33258 staining kit (cat. C1018) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Annexin V-FITC apoptosis detection kit (cat. A211) and Cell Cycle assay kit (cat. A411) were purchased from Vazyme Biotech Co., Ltd. (Jiangsu, China). The primary antibodies for β-actin, Bcl-XL, Bax, Bak, caspase-3 as well as c-caspase-3 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The horseradish peroxidase (HPR)-linked goat anti-rabbit IgG secondary antibodies were purchased from Bio-Rad ( Hercules, CA, USA).

Cell culture. CNE-2 cell line (a human NPC cell line) was obtained from the Institute of Biochemistry and Molecular Biology, Guangdong Medical College (Guangdong, China). Cells were cultured in RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL) and 100 µg/ml penicillin-streptomycin (Beyotime Institute of Biotechnology), and maintained at 37°C in a humidified atmosphere of 5% CO₂.

WST-8 conversion assay. The effect of Na₂SeO₃ on cell viability/proliferation was determined using the WST-8 assay. Briefly, CNE-2 cells were seeded into 96-well plates at a density of 5,000 cells/well and incubated for 24 h. Then the cells were treated with Na₂SeO₃ at different concentrations (0, 2, 5, 10, 20, 50 and 100 µM) for 0, 24, 48, 72 or 96 h. WST-8 conversion was then assessed using a one-step CCK-8, according to the manufacturer's instructions. All tests were carried out in triplicate. The absorbance was measured at 450 nm by using a Synergy multifunctional microplate reader (Bio-Rad) with a reference at 650 nm serving as blank. The inhibiting ability of Na₂SeO₃-treated cells was calculated as the percentage of inhibition compared to untreated cells, which were arbitrarily assigned 100% viability. GraphPad Prism 4.0 was used to analyze the IC₅₀ of Na₂SeO₃ in this case.

Cell cycle analysis. After treated with Na₂SeO₃ (0, 5, 10 and 20 µM) for 6 h, CNE-2 cells were collected and fixed overnight in 75% cold ethanol at -20°C. The cells were then washed twice with cold PBS (pH 7.4) and stained with Cell Cycle assay kit according to manufacturer's instructions. Cell cycle distribution was determined using a flow cytometer (Beckman Coulter Epics XL-MCL; Beckman Coulter, Miami, FL, USA) and analyzed using CellQuest software (18).

DAPI staining. CNE-2 cells at a density of 1x10⁵ were grown overnight in a cell culture dish. Then the cells were incubated with Na₂SeO₃ (0, 5, 10 and 20 µM) for 3 h. After incubation, cells were washed with ice-cold PBS and stained with DAPI staining fluid for 20 min in the dark at room temperature. After that, the cells were washed twice with PBS. Cell images were captured using a fluorescence microscope (Nikon Corp., Tokyo, Japan).

Hoechst 333258 staining. After treatment with Na₂SeO₃ at a series of concentration for 3 h, the CNE-2 cells were fixed and washed twice with PBS, then incubated with Hoechst 333258 for 30 min at room temperature. Then the cells were washed with PBS for three times, and the changes of nucleic morphologies were observed under fluorescence microscopy (Inverted Biological Binocular Microscope; Nikon Corp.).

DNA fragmentation analysis. CNE-2 cells (3x10⁶) were incubated with Na₂SeO₃ (0, 5, 10 and 20 µM) for 6 or 12 h and then harvested. The characteristic ladder pattern of DNA breakage was analyzed by agarose gel electrophoresis using an Apoptosis DNA Ladder detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The DNA preparations were electrophoresed in 1% agarose gel, then stained with ethidium bromide and observed under UV transilluminator (ChemiDoc XRS Syngene; Bio-Rad).

Annexin V-FITC apoptosis detection. Apoptosis was also analyzed by utilizing an Annexin V-FITC apoptosis detection kit (Vazyme Biotech Co., Ltd.). Briefly, cells were seeded in 100 mm-well plates and incubated for 24 h and then treated with Na₂SeO₃ (0, 5, 10 and 20 µM) for 24 h. After treatment, ~1x10⁶ cells were harvested, washed twice with PBS, and stained with Annexin V-FITC and PI according to the manufacturer's instructions. The resulting fluorescence was detected by flow cytometer ( Beckman Coulter Epics XL-MCL, Beckman Coulter) with CellQuest analysis software.

Measurement of intracellular active oxygen. Formation of intracellular ROS was determined using a fluorescent probe 2',7-dichlorofluorescein diacetate (Beyotime Institute of Biotechnology). DCFH-DA, a non-fluorescent substance, can cross to cell membranes and be hydrolyzed by intracellular peroxides. CNE-2 cells were incubated with Na₂SeO₃ for 3 h, followed by another 30-min incubation with 10 µM DCFH-DA. Then the cells were washed with PBS three times and the changes of fluorescence were observed using fluorescence microscopy.

Measurement of MMP. The MMP was determined using the mitochondria-specific lipophilic cationic fluorescence dye JC-1 detection kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology). In brief, CNE-2 cells were seeded in 6-well culture plates at a density of 3x10⁵ cells/well and cultured with or without Na₂SeO₃ (0, 5, 10 and 20 µM) for 3 h, followed by a 30-min incubation with 10 µM JC-1 in the dark, then the cells were washed twice with...
PBS, and the fluorescent intensity was examined by a fluorescence microscope. In healthy cells with high MMP, JC-1 gathers in mitochondrial matrix as J-aggregates, which emits red fluorescence (normal membrane potential). When the MMP collapses, the JC-1 cannot accumulate in mitochondria and are converted to monomer which can emit green fluorescence (declined membrane potential).

**Western blotting.** Cells were washed three times with ice-cold PBS. Cell lysates were prepared with RIPA buffer (Beyotime Institute of Biotechnology) containing 150 mM NaCl, 1% Triton X-100, 20 mM Tris (pH 7.5) and 1% of two kinds of protein inhibitors including protein phosphatase inhibitor and phenylmethanesulfonyl fluoride (PMSF). After a forced vortex, cell lysates were incubated on ice for 2 h, and centrifuged at 12,000 rpm for 15 min at 4°C to remove insoluble debris. Protein concentrations were determined using the BCA method (Beyotime Institute of Biotechnology). The whole cell lysates were treated by boiling in loading buffer containing SDS and electrophoresised in SDS-PAGE, then transferred onto membranes (Immobilon-P; Millipore, Billerica, MA, USA). After blocking with 5% skim milk for 1 h, the membranes were incubated with specific primary antibodies at 4°C overnight, followed by incubation with enzyme-linked secondary antibodies for 2 h at room temperature. The membranes were then visualized by enhanced chemiluminescence (ECL), and the result was analyzed by ChemiDoc XRS transilluminator (both from Bio-Rad). The gray analysis was carried out by ImageJ Gray Analysis software.

**Statistical analysis.** Results are reported as mean ± SD of triplicate independent experiments. Statistical analyses were performed with the SPSS v.19.0 software and performed by one-way analysis of variance (ANOVA). P<0.05 for each concentration versus control was considered to indicate significance.

**Results**

Na2SeO3 inhibits proliferation of CNE-2 cells. We first observed the effect of Na2SeO3 on proliferation of CNE-2 cells. As shown in Fig. 1A, Na2SeO3 significantly inhibited proliferation of CNE-2 cells in a time- and dose-dependent manner. At 20 μM, Na2SeO3 inhibited proliferation of CNE-2 cells by 5.55, 29.50, 46.11 and 53.19% after treatment for 24, 48, 72 and 96 h, respectively. When the concentration of Na2SeO3 increased to 100 μM, the inhibition rate...
reached 77.20% after treatment for 96 h. The IC_{50} of Na_{2}SeO_{3} for treating CNE-2 cells for different time periods were analyzed by the curve fitting (Fig. 1B). As compared with the 24 and 72 h, the fitting degree of 48 and 96 h were more accurate, and the IC_{50} was 19.86 and 11.9 µM, respectively. Based on these results, Na_{2}SeO_{3} was used at 5, 10 and 20 µM in the following experiments. In addition, after treatment with Na_{2}SeO_{3}, the cell morphology was observed under a microscope. As shown in Fig. 1C, after 24 h treatment with Na_{2}SeO_{3}, the cell density was significantly decreased in a dose-dependent manner. The cells shrunk, retracted from neighboring cells, lost their flat and polygonal shape, and ultimately detached from the culture dish, indicative of a cell death induced by Na_{2}SeO_{3}.

Na_{2}SeO_{3} induces cell cycle arrest in CNE-2 cells. We next observed the effect of Na_{2}SeO_{3} on cell cycle of CNE-2 cells. CNE-2 cells were treated with Na_{2}SeO_{3} at a series of concentrations for 6 h followed by PI staining and analyzed by a flow cytometer. As shown in Fig. 2A and B, treatment of CNE-2 cells with Na_{2}SeO_{3} at relatively higher concentrations (10 and 20 µM) resulted in a significant accumulation of cells at G_{0}/G_{1} phase (p<0.05); a sub-G_{1} apoptotic peak was also observed, while at a relatively lower concentration (5 µM), Na_{2}SeO_{3} induced S phase arrest (p<0.05). Together, these findings suggest that Na_{2}SeO_{3} could induce cell cycle arrest in CNE-2 cells.

Na_{2}SeO_{3} induces apoptosis in CNE-2 cells. The cell cycle arrest as well as the apoptotic peak induced by Na_{2}SeO_{3} led us to further determine if Na_{2}SeO_{3} could induce apoptosis of CNE-2 cells using an Annexin V-FITC apoptosis detection kit. Na_{2}SeO_{3} treated or untreated cells were analyzed by flow cytometry using Annexin V-FITC/PI double staining assay. The apoptotic cells could be divided into early-stage apoptosis (Annexin V+ and PI−) and late-stage apoptosis (Annexin V+ and PI+), which are shown in the lower right (LR) and upper right (UR) quadrants of the FACS histograms, respectively (Fig. 3A, left panel). As shown in Fig. 3B, the percentage of total apoptotic cells in CNE-2 cells was 5.2% in control cells (early-stage: 1.3% and late-stage: 3.9%), 9.3% in cells treated with 5 µM Na_{2}SeO_{3} (early-stage: 6.0% and late-stage: 3.3%), 51.2% in cells treated with 10 µM Na_{2}SeO_{3} (early-stage: 50.3% and late-stage: 0.9%) and 60.6% in cells treated with 20 µM Na_{2}SeO_{3} (early-stage: 60.0% and late-stage: 0.6%). These results indicate that Na_{2}SeO_{3} significantly induced apoptosis (including early- and late-stage apoptosis, p<0.01) in CNE-2 cells.

To further confirm Na_{2}SeO_{3}-induced apoptosis, we next observed the morphological change of cell nuclei induced by Na_{2}SeO_{3} via staining cell nuclei with DAPI and Hoechst 333258. As shown in Fig. 3C, chromatic agglutination and karyopyknosis were observed after treatment with Na_{2}SeO_{3} (5, 10 and 20 µM) for 3 h, and fragmented nuclei were observed after treatment for 6 and 12 h. In contrast, cells in control group exhibited normal intact nuclei. Furthermore, after treatment with Na_{2}SeO_{3}, a series of DNA ladders were observed (Fig. 3D), indicative of late-stage apoptosis in CNE-2 cells. These results further demonstrated that Na_{2}SeO_{3} can induce significant apoptosis in CNE-2 cells.

Na_{2}SeO_{3} downregulates Bcl-XL and upregulates Bak and Bax. The Bcl-XL, Bak and Bax proteins play crucial roles in the regulation of apoptosis (19,20). We examined the changes in the expression of Bcl-XL, Bak and Bax by western blotting in CNE-2 cells in response to Na_{2}SeO_{3} treatment. The representative blots for CNE-2 cells are shown in Fig. 4A, and the relative expression of these proteins was also calculated...
through normalization to β-actin expression and is summarized in Fig. 4B. The results showed that after treatment with Na₂SeO₃ (5, 10 and 20 µM) for 6 h, the expression of Bak and Bax was increased and the expression of Bcl-XL was decreased in a dose-dependent manner in CNE-2 cells (Fig. 4). These data suggest that Na₂SeO₃ might induce CNE-2 cell apoptosis through downregulation of Bcl-XL and upregulation of Bak and Bax.

Na₂SeO₃ induces disruption of MMP in CNE-2 cells. Mitochondria play a key role in cell apoptosis and depletion of MMP is one of the early and key events that occur following induction of cellular apoptosis. To determine the changes of MMP in CNE-2 cells after Na₂SeO₃ treatment, JC-1 staining was carried out. The fluorescence microscopy observation confirmed that Na₂SeO₃ (5, 10 and 20 µM)-treated cells showed a progressive loss of red J-aggregates fluo-
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rescence and appearance of green monomer fluorescence in the cytoplasm, and this decrease obviously occurred in a dose-dependent manner (Fig. 5). These data suggest that the intrinsic mitochondrial pathway of apoptosis might be one of the mechanisms involved in cell death of CNE-2 cells induced by Na$_2$SeO$_3$.

Na$_2$SeO$_3$ increases caspase-3 activity in CNE-2 cells. It is well known that the intracellular translocation of Bak and Bax can induce the loss of MMP, which is linked to the initiation and activation of the apoptotic process in cells (21,22). Due to the loss of MMP, cytochrome c is released into the cytosol from mitochondria, which activates pro-caspase-9 in the apoptosome and leads to the cleavage of caspase-3 (1). Subsequently, active cleaved caspase-3 can cleave a broad spectrum of target proteins and finally result in apoptotic cell death. To examine whether apoptosis induced by Na$_2$SeO$_3$ involves caspase activation, the total and cleaved caspase-3 were examined by western blotting. The results showed that treatment with Na$_2$SeO$_3$ for 6 h resulted in significant increase in caspase-3 activity in a dose-dependent manner (Fig. 6) as indicated by increase of cleaved caspase-3 and decrease of total PARP, a substrate of caspase-3, suggesting that apoptosis of CNE-2 cells induced by Na$_2$SeO$_3$ might involve the activation of caspase-3 pathway.

Discussion

 Compared to other types of head and neck cancer, NPC is a highly metastatic disease. Despite advances in diagnosis and treatment, the 5-year survival of this malignant disease remains disappointing due to the local recurrence/metastasis and resistance to chemo- and radiotherapies. Therefore, an increasing understanding of the complex metastases/recurrence mechanisms of NPC is imperative to the development of more effective mechanism-based therapeutic modalities for this malignancy.

Se is an essential trace element because of its role in glutathione peroxidase (GSH-Px), the daily dietary supply of Se in human body must reach 50 µg according to the Chinese Nutrition Society, a standard that has been adopted
Figure 5. CNE-2 cells were treated with the indicated doses of sodium selenite (Na₂SeO₃) for 3 h and then harvested, stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye, and finally analyzed by fluorescence microscope. Green fluorescence means that Na₂SeO₃ resulted in a significant loss of mitochondrial membrane potential (MMP) in CNE-2 cells (magnification, x200).

Figure 6. The effect of sodium selenite (Na₂SeO₃) on the expression of PARP, caspase-3 and c-caspase-3 in CNE-2 cells. Treatment and cells as shown in Fig. 4. (A) Treatment of CNE-2 cells with Na₂SeO₃ resulted in a reduction of PARP expression and an increase in caspase-3 and c-caspase-3 expression. (B) The relative expressions of PARP, caspase-3 and c-caspase-3 proteins in CNE-2 cells were calculated based on β-actin expression, which was used as loading control. Representative blots are shown from three independent experiments, and values are expressed as the mean ± SD. *P<0.05 vs. control.

Figure 7. CNE-2 cells were treated with the indicated doses of sodium selenite (Na₂SeO₃) for 3 h and then harvested, stained with DCFH-DA dye, and finally analyzed by fluorescence microscopy. Fluorescence shows that Na₂SeO₃ resulted in a significant increase of reactive oxygen species (ROS) in CNE-2 cells (magnification, x200).
by the World Health Organization (Geneva, Switzerland) (26). In addition to its nutritional functions, accumulating evidence has shown that super-nutritional selenite intake has antitumor activity both in vitro and in vivo (14,27-32). Mechanistically, Na2SeO3 can induce cell apoptosis through mitochondrial apoptotic pathway in a variety of cancer cell lines including ovarian carcinoma, lung carcinoma, colon cancer and breast cancer cells (15,33-36). In the present study, we demonstrated for the first time to our knowledge that Na2SeO3 can inhibit proliferation and induce cell cycle arrest in CNE-2 NPC cells. Interestingly, treatment of CNE-2 cells with higher concentrations of Na2SeO3 resulted in a significant accumulation of cells at G0/G1 phase, while treatment with the agent at lower concentrations arrested the cell cycle at S phase. Therefore, further studies are warranted to elucidate the detailed underlying mechanisms.

Mitochondrial pathway is critical in cell apoptosis. Mitochondrial dysfunction can induce the activation of caspase-9 and the subsequent caspase-3, followed by cleavage of its substrates such as PARP, eventually inducing cell apoptosis (4,5). Increase of ROS production could induce mitochondrial dysfunction. Our studies show that Na2SeO3 could increase ROS production, induce disruption of MMP and activate the caspase-3 pathway. All these data suggest that Na2SeO3-induced apoptosis in CNE-2 cells might be associated with the mitochondrial apoptotic pathway. This was further confirmed by the results that Na2SeO3 could downregulate Bcl-XL and upregulate Bak and Bax, which are apoptosis-related proteins located in mitochondria. Therefore, we speculated that Na2SeO3 inhibited CNE-2 cell growth through its effects on mitochondria.

In summary, our studies demonstrate that Na2SeO3 has significant anti-proliferation- and apoptosis-induction effects in CNE-2 cells by cell cycle arresting and regulation of mitochondria-mediated intrinsic caspase pathway, suggesting that Na2SeO3 might have potent therapeutic potentials in the treatment of NPC.

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