Original Article

Tin (IV) Oxide (SnO_2) Nanoparticles Inhibit the Viability of Cervical Cancer HeLa Cells Through Induction of Apoptosis

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Abstract

Introduction: Resistance to chemotherapy and severe side effects have been reported as the main reasons for treatment failure in patients with cervical cancer. Therefore, it is necessary to find new treatment strategies with fewer side effects and more efficacy. This study aimed to investigate the cytotoxic property of tin (IV) oxide (SnO_2) nanoparticles (NPs) against human cervical cancer cells (HeLa cells). In addition, the molecular mechanism of anticancer activity of SnO_2 NPs was evaluated. **Materials and Methods:** The cytotoxicity of SnO_2 NPs against HeLa cells and normal mouse fibroblast cells (L929) was studied using an MTT assay. To determine the mechanism of action of SnO_2 NPs, the cells were treated with the half maximal inhibitory concentration values of SnO_2 NPs for 24 h and apoptotic cell percentage was assessed by Annexin-PI and flow cytometry. In addition, real-time quantitative polymerase chain reaction (PCR) was used to evaluate the mRNA expression levels of apoptotic genes (Bax and Bcl-2). **Results:** SnO_2 NPs suppress the viability of HeLa cells in a dose-dependent manner. This compound was more cytotoxic against HeLa cells than L929 cells. Flow-cytometry analysis revealed that SnO_2 NPs significantly caused cell growth arrest. Moreover, real-time PCR results showed that SnO_2 NPs treatment decreased Bcl-2 and increased Bax expression level. **Conclusion:** SnO_2 NPs treatment significantly inhibit HeLa cells viability through the induction of apoptosis. Interestingly SnO_2 NPs were more cytotoxic against HeLa cells than normal fibroblast cells, which may provide promising evidence for their applications as an anticancer drug.

Keywords: *Apoptosis, cervical cancer, nanoparticles, SnO_2 NPs*

Introduction

Cervical cancer is one of the most frequent gynecological cancers and ranks second in cancer incidence among women worldwide.[1] Almost all types of cervical cancer—squamous cancer, adenosquamous cancer, and adenocarcinoma—are now believed to be associated with human papillomavirus (HPV) as the most widespread human viral infection.[2,3] Using oral contraceptives, starting sexual activity at an early age, different sexual partners, genital warts, and tobacco smoking are some risk factors associated with cervical cancer.[4,5]

Thirteen percent of patients who suffer from cervical cancer are diagnosed at advanced stages with a 5-year survival rate of 16.5% in comparison to 91.5% for a localized form of cervical cancer.[4,6,7] Surgery, radiotherapy (RT), and chemotherapy are conventional therapy for patients at an early stage or localized form of cervical cancer however patients suffering from metastatic cervical cancer have failed to receive standard therapy due to their clinical heterogeneity.[7] Thus, a new alternative therapy is crucial because of side effects, low efficacy, and resistance to current therapies.[8]

Recently, researchers have paid much attention to developing new drugs against cancers that target apoptotic pathways.[9] Apoptosis in multicellular organisms plays a regulatory role in tissue homeostasis and cell proliferation. Cell death regulation dependent on the ratio of pro-and anti-apoptotic proteins and disruption of these protein balance has been said to exert a pivotal role in cancer pathogenesis.[9]

Capability to create nanoparticles (NPs) is one of the major reasons that special attention is paid to nanotechnology.[10,11] Targeted drug delivery systems along with improved bioavailability are the main features of NPs; thus, they could offer promising tools to develop newer treatment platforms for cancers.[12]

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Investigation of possible effects of metal oxide NPs including cytotoxicity, genotoxicity, inflammation, and apoptosis on cancerous cells through the generation of reactive oxygen species (ROS) and oxidative stress have been mentioned in the previous report.[11]

Tin (IV) oxide (SnO_2) is one of the semiconducting NPs with a wide bandgap energy potential (3.6 eV).[13] In recent years, SnO_2 NPs have received attention for numerous biomedical applications and show antimicrobial and antioxidant activities.[11] Ahamed *et al.* have revealed that the SnO_2 NPs significantly enhances cell death in human breast cancer cells through induction of oxidative stress.[11] Moreover, Tammina *et al.*[14] and Roopan *et al.*[13] have investigated the anticancer property of SnO_2 NPs against human cell lines including colorectal (HCT116), lung (A549), and hepatocellular (HepG2) cancer cells. However, studies on the toxicity effects of SnO_2 NPs on cervical cancerous cells are largely lacking. Here for the first time, the anticancer effects of SnO_2 NPs against cervical cancer cells were evaluated. In addition, the potential molecular mechanism of the cytotoxic property of SnO_2 NPs was studied.

Materials and Methods

SnO_2 NPs characterization

SnO_2 NPs with 18 nm diameter, purity of 99.9%, were provided by Pishgaman Nanomaterial Company (Mashhad, Iran). The characteristics of SnO_2 NPs were carried out with transmission electron microscopy and X-ray diffraction by US Research Nanomaterials.

Cell lines and culture conditions

Human cervical cancer (HeLa) cell line and mouse fibroblast cell line L929 (as noncancerous control cells) were purchased from the National Cell Bank of Pasteur Institute (Tehran, Iran) and cultured in RPMI1640 medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin and maintained at 37°C in an atmosphere of 5% CO_2. All reagents used for cell culture were obtained from Gibco (Thermo Fisher Scientific, Waltham, Massachusetts).

Cell viability assay

The cytotoxic effect of SnO_2 NPs on HeLa and L929 cell lines was determined using an MTT assay.

Cells were cultured in 96-well plates at a density of 1 x 10^4 cells/well and after 24 h, the medium replaced with fresh medium supplemented with specified concentrations of SnO_2 NPs (0.78, 1.5, 3.12, 6.25, 12.5, 25, 50, 100, and 200 µg/mL). In order to detect cell viability after 24h, 20 c MTT reagent was added to each well at a final concentration of 0.5 mg/mL and maintained in an incubator for 4 h at 37°C with 5% CO_2 in a humidified atmosphere. Following removal of the culture medium, 150 µL DMSO/well was added to dissolve MTT crystals. In the control wells, the complete medium without drug was added to the cells. The absorbance of the samples at 570 nm (OD570) was read using an Elisa reader (Stat fax, Palm City, FL, USA). The formula: cell viability = OD570 (sample)/OD570 (control) ×100 was used for the calculation of cell viability. The half maximal inhibitory concentration (IC_{50}) value related to the cytotoxicity of the drug was calculated using GraphPad Prism software, version 8.00 (GraphPad Software, San Diego, California) using a nonlinear regression and dose-response models.

RNA extraction and real-time quantitative polymerase chain reaction assay for mRNA expression detection

We used 6-well plates to culture cervical cancer cells and then treated with the IC_{50} of SnO_2 NPs for 24 h. After treatment, RNX solution (CinnaGen, Iran) was used to extract total RNA according to the manufacturer's protocol. Checking the quality and quantity of RNA were determined using agarose gel electrophoresis and a photo nanometer (IMPLEN GmbH, Germany), respectively. cDNA was synthesized using 1 µg of total RNA by a Revert AidTM First Strand cDNA Synthesis Kit (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). Quantitative polymerase chain reaction (qPCR) assays for the determination of mRNA expression level of Bax, Bcl-2, and GAPDH (internal control) were performed in duplicate using an ABI 7300 (Applied Biosystems, Foster City, California). Amplifications were done in 20 µL mixtures of 1 µL cDNA, 1 µL of 10 µM primers (Table 1 represents the sequences of the primers), and 10 µL SYBR Green PCR master mix (Applied Biosystems, Warrington, UK). The thermocycling conditions were 95°C for 10 min for initial denaturation, and 95°C for 15 s, annealing, and extension at 60°C for 60 s for 40 cycles. $2^{-\Delta\Delta Cq}$ method was used to calculate the relative amount of mRNA[15] and normalized to the level of GAPDH.

Flow cytometry for apoptosis detection

Annexin V-FITC-PI kit (Apoptosis detection kit, Roch, Germany) was used to measure the number of apoptotic cell death by flow cytometry. 10^5 cells were incubated with the IC_{50} of SnO_2 NPs for 24 h at 37°C. Then cells were harvested, washed twice with cold PBS and, resuspended in 200 µL binding buffer. The cells were then stained with 5 µL Annexin-V and PI for 20 min in the dark at room temperature, and subjected to analysis by Partec PAS-II flow cytometer

Table 1: Primer sequences used for RT-qPCR

Genes	Forward primer	Reverse primer
Bcl-2	5′-TGTGGATGACTGAGTACCTGAACC-3′	5′-CAGCCAGGAGAAATCAAACAGAG-3′
Bax	5′-TTGCTTCAGGGTTTCATCCAG-3′	5′-AGCTTCTTGGTGGACGCATC-3′
GAPDH	5′-CGTCTGCCCTATCAACTTTCG-3′	5′-CGTTTCTCAGGCTCCCTCT -3′

(Partec, Munster, Germany). In order to analyze the data, FloMax 1.0 software was used.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) software program, version 16.0 (SPSS, Chicago, Illinois) was used for data analysis. All results were expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) was done for group comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of SnO_2 NPs treatment on viability of cell lines

The viability of HeLa and L929 cell lines was detected using an MTT assay after treatment with different concentrations (0.78–200 µg/mL) of SnO_2 NPs for 24 h. In Figure 1, the pattern of HeLa and L929 cells response to the cytotoxic effect of SnO_2 NPs is presented. Our results indicated that SnO_2 NPs treatment was able to inhibit the viability of HeLa and L929 cells in dose-dependent manners. The IC_{50} value of SnO_2 NPs for HeLa cells was 15.290 µg/mL and for L929 was 30.426 µg/mL. Figure 2 shows that SnO_2 NPs at a concentration of 3.125 µg/mL and more could significantly reduce HeLa cells viability in comparison to nontreated cells. On the contrary, for L929 cells, higher concentration of SnO_2 NPs (at least 6.12 µg/mL and more) is required to reduce significantly cell viability in comparison to nontreated cells. Exposing of cells to different concentrations of SnO_2 NPs shows more cytotoxicity against HeLa cells than control L929 cells. For example, treatment of HeLa and L929 cells with 200 µg/mL of SnO_2 NPs reduced cell viability to 15% and 30% of control, respectively.

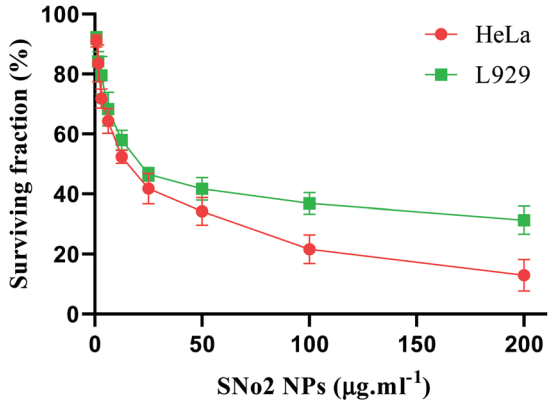

Figure 1: Sensitivity of HeLa and L929 cell lines to SnO_2 NPs. MTT assay was used to determine cell viability following treatment of cells with specified concentrations of SnO_2 NPs for 24 h. Results were presented as the mean ± standard deviation

Effects of SnO_2 NPs treatment on mRNA expression levels of Bax and Bcl-2 in cervical cancer cells

To determine the possible mechanism of SnO_2 NPs cytotoxicity, Bax and Bcl-2 mRNA expression levels in HeLa cells were detected using real-time qPCR (RT-qPCR) assay following 24 h of SnO_2 NPs treatment. As illustrated in Figure 3, compared with the untreated cells, the expression level of Bax mRNA was dramatically increased ($P < 0.001$), whereas the expression level of Bcl-2 mRNA decreased in a significant manner ($P < 0.05$).

Flow-cytometry analysis of apoptosis following treatment of cell with SnO_2 NPs

Annexin V/PI test was carried out for the determination of the mode of cell death after exposure of cells to IC_{50} value of SnO_2 NPs for 24 h. Apoptotic and necrotic incidence in HeLa cancer cell line was determined by flow cytometry. As shown in Figure 4, the apoptotic and necrotic incidence was 35.62% and 6.61%, respectively. In contrast, 98.96% of untreated cells were intact.

Discussion

Despite several preventative and therapeutic methods, the survival rate still remains poor among cervical cancer patients.

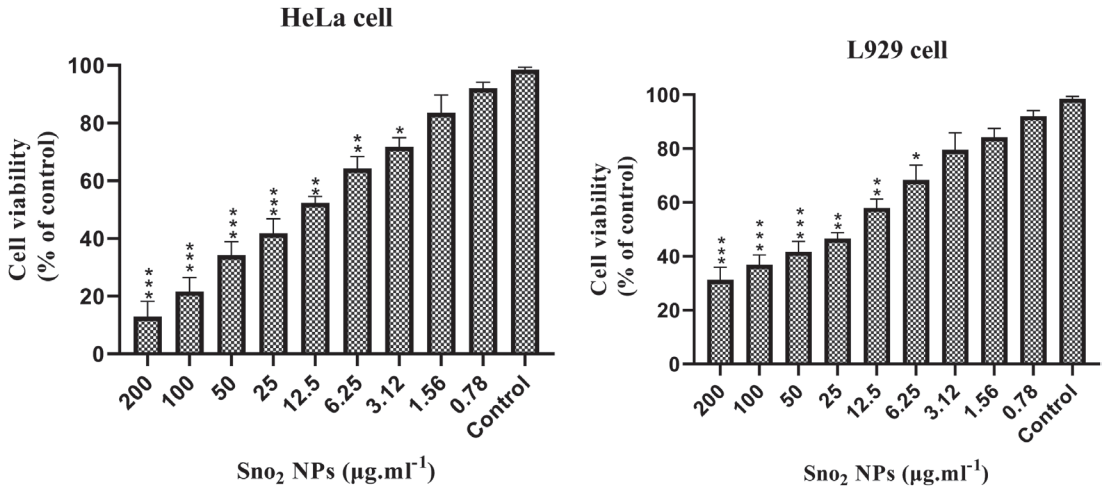

Figure 2: The pattern of HeLa and L929 cells response to the cytotoxic effect of graded concentration of SnO_2 NPs for 24h ($P < 0.001$*, $P < 0.01$ **, $P < 0.05$ * as compared to control)**

Resistance to chemotherapy and several side effects have been reported as common reasons for treatment failure in patients with cervical cancer. Therefore, it is vital to develop an effective and low-toxicity anticancer therapy for cervical cancer.[16,17]

SnO_2 NPs are one of the promising NPs used in nanomedicine because of their unique features.[11] Here for the first time, the effect of SnO_2 NPs on cervical cancer cells was investigated.

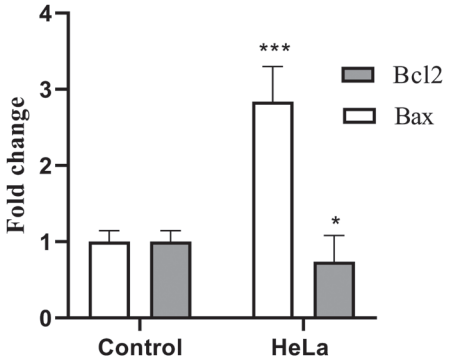

Figure 3: Effects of SnO_2 NPs on mRNA expression levels of Bax and Bcl-2 in cervical cancer cell line. Cells were treated with the IC_{50} value of SnO_2 NPs for 24 h and then total RNA was extracted and used for RT-qPCR. Results are expressed as the mean ± SD ($P < 0.05$ *, $P < 0.001$ * as compared to control)**

As the results show, SnO_2 NPs could exert a cytotoxic effect on HeLa cell line in a dose-dependent manner. Interestingly, the cytotoxicity of SnO_2 NPs was more pronounced on HeLa cells than control noncancerous dermal fibroblast cells. In fact, a higher concentration of SnO_2 NPs was required to induce cell death in control than cervical cancer HeLa cells, indicating a higher sensitivity of cervical cancer cell lines against SnO_2 NPs.

In previous works, the cytotoxicity of SnO_2 NPs against human breast (MCF-7), lung (A549), liver (HepG2), and colorectal (HCT116) cells was studied.[11,13,14] However, no data were available regarding the effect of SnO_2 NPs on cervical cancer cells. Ahamed *et al*.[11] showed that SnO_2 NPs could induce a cytotoxic effect on MCF-7 cells in a dose and time-dependent manner after treated with different concentrations of SnO_2 NPs (5–200 µg/mL). In their study, it has been shown that SnO_2 NPs were significantly induced cytotoxicity in breast cancer MCF7 cells in comparison to normal human lung fibroblast cells which suggested their capability to target cancerous cells selectively.

The reason for this unique characteristic of NPs such as SnO_2 was not completely understood. However, it has been shown that SnO_2 treatment induces ROS production and cancer cell

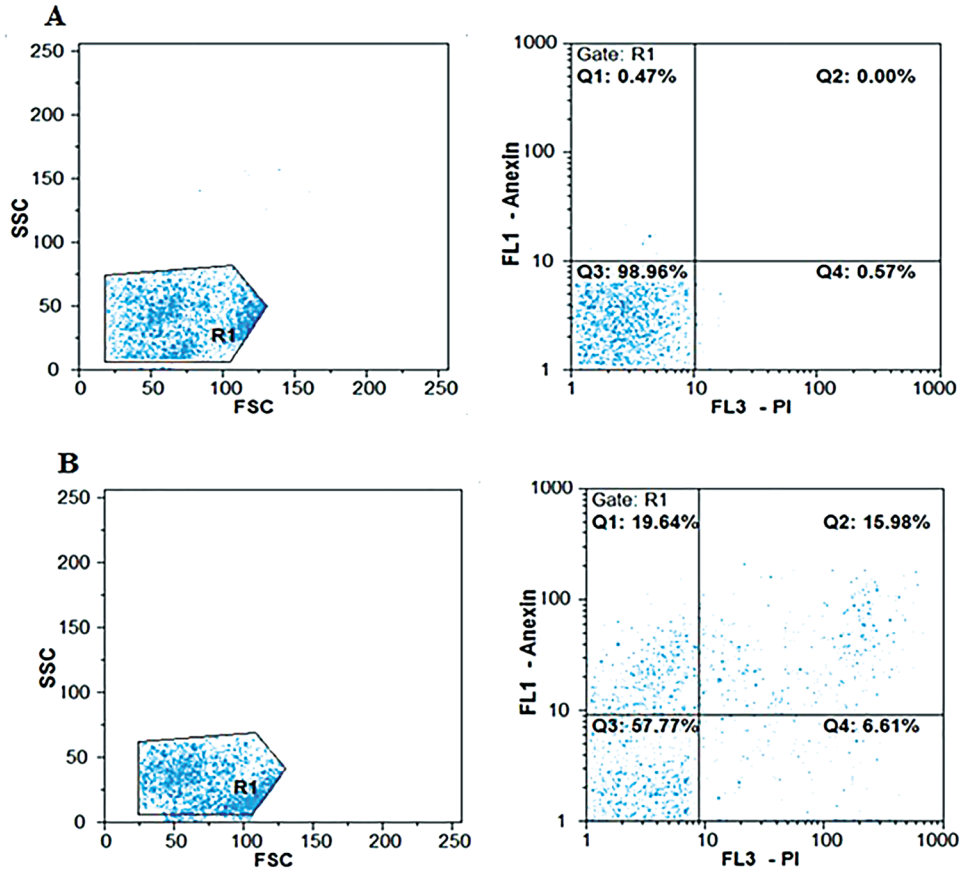

Figure 4: Determination of the mode of HeLa cells death using Annexin/ PI staining and flow cytometry. The diagram shows the status of the (A) cells after exposure to the IC_{50} concentrations of SnO_2 NPs and implies that SnO_2 NPs can induce both apoptosis and necrosis in cervical cancer cell line but (B) untreated cells as a control was intact

was shown to be more vulnerable to ROS toxicity than a normal cell.[18] In addition because of leaky tumor vasculature, NPs can more easily penetrate the endothelium of tumor tissue than normal tissue. Therefore, it will aggregate and trap in the tumor site, leading to enhanced retention of NPs in the tumor and consequently elicit a number of biological stress responses, including mitochondrial dysfunction, oxidative stress, cell cycle arrest, and apoptosis.[19]

Induction of cancer cell apoptosis is a key characteristic of chemotherapy drugs. Apoptotic markers such as loss of mitochondrial membrane potential and disturbance of cell cycle were reported for the cells to expose to SnO_2 NPs.[11,20,21] Also, cell volume loss, considerable swelling of the cells, and chromatin condensation were proposed as a morphological alteration in the HepG2 cell line that was treated with SnO_2.[13]

Several studies have been described that nanomaterials exert their toxicity through membrane damage and cell death by ROS formation.[22-24] In this regard, Tammina *et al*.[14] have proposed ROS formation and apoptotic cell death could be the cause of SnO_2 NPs' toxicity against A549 and HCT116 cancer cells.

Our flow-cytometry analysis using Annexin-PI implied that the SnO_2 NPs induce apoptosis in cervical cancer cells [Figure 4]. Treatment of HeLa cells with the IC_{50} value of SnO_2 NPs induces apoptosis in a high percentage of cells (35.6%). However, our results show that a small percentage of cells (6.61%) died through necrosis. To further confirmed the molecular mechanism of cell death, HeLa cells were exposed to SnO_2 NP_S, and then qPCR was used to evaluate the mRNA expression level of two important apoptotic genes including Bax and Bcl-2.

Dysregulation of Bcl-2 as an anti-apoptotic gene and Bax as a pro-apoptotic gene plays a key role in cancer progression. Several studies have claimed that down-regulation of Bcl-2 and upregulation of Bax may lead to dysfunction of mitochondria and cause apoptosis.[25-27]

In our work, SnO_2 NPs treatment of HeLa cells markedly increases Bax expression level, whereas SnO_2 NPs treatment of HeLa cells decreases the Bcl-2 expression level. Following exposure of cells to SnO_2 NPs, the ratio of Bcl-2/Bax dramatically decreases, indicating the occurrence of apoptosis. Therefore, the results of the flow cytometry and qPCR assay show that the cytotoxicity of SnO_2 NPs against HeLa cells was related to the induction of apoptosis.

Previous studies[22-24] suggested that cell death by NPs such as SnO_2 NPs[14] in different cancer cells is due to ROS-mediated membrane damage. Therefore, it can be postulated that in our study the cytotoxic property of SnO_2 NPs against HeLa cells may be related to induction of ROS level. It is worth mentioning that high levels of ROS can be the reason for mitochondrial membrane damage which is lead to its depolarization. This event causes destabilization of Bcl-2 and lowering the ratio of Bcl-2 to Bax and ultimately enhanced apoptosis.[28]

There is no investigation regarding the effect of SnO_2 NPs on Bax and Bcl-2 genes expression, however previous studies showed other NPs exerted their cytotoxic effects on cervical cancer cells through upregulation of Bax and downregulation of Bcl-2 mRNA and protein expression.[29-31]

Conclusion

Taken together, our findings show that SnO_2 NPs treatment significantly inhibit HeLa cells viability through induction of apoptosis. Interestingly our results show that SnO_2 NPs were more cytotoxic against HeLa cells than normal fibroblast cells, which may provide promising evidence for its application as an anticancer drug.

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Nil.

Conflicts of interests

There are no conflicts of interest

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