Zinc enhances chemosensitivity to paclitaxel in PC-3 prostate cancer cells

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Abstract. Paclitaxel-based chemotherapy is a promising approach for prostate cancer treatment. However, single-drug chemotherapy is associated with an increased risk of drug resistance. Therefore, novel combination chemotherapy regimens are a popular topic of research. Zinc participates in the regulation of apoptosis, for example in the form of Zn⁺⁺ and via zinc-dependent enzymes. Zinc can either induce or suppress apoptosis, and its effect depends primarily on its concentration. Previous research has demonstrated that physiological concentrations of zinc can directly induce apoptosis of PC-3 prostate cancer cells via the mitochondrial pathway. In prostate cancer tissues, zinc concentrations have been demonstrated to be reduced compared with non-cancerous tissues. Furthermore, the concentration of zinc has been demonstrated to decrease further with cancer progression. In the present study, it was investigated whether exposure of PC-3 cells to zinc improved their sensitivity to the chemotherapeutic agent, paclitaxel. MTT assays, cell clone formation assays, Hoechst staining and flow cytometry revealed that zinc enhanced PC-3-cell chemosensitivity to paclitaxel. Western blotting and reverse transcription-polymerase chain reaction were used to determine that the mitochondria-mediated apoptosis signaling pathway is involved with zinc/paclitaxel-induced cell death. The present study provides a foundation for the development of novel tumor combination therapy.

Introduction

Prostate cancer is among the most common types of cancer in elderly men, and is a leading cause of cancer-associated mortality in Western countries (1). Treatment strategies for prostate cancer include surgery, hormone therapy, radiotherapy and chemotherapy (2). Chemotherapy serves an important role in the treatment of castration-resistant metastatic prostate cancer (3). Paclitaxel has been demonstrated to result in significant antitumor responses in combination with other agents (4,5). However, there are limitations to chemotherapy, including the development of drug resistance, particularly multidrug resistance (6). Therefore, the identification of effective mechanisms to prevent chemotherapy resistance is essential.

The mechanism by which paclitaxel acts on prostate cancer cells is different from the mechanisms of action of other anticancer drugs, and it functions by inhibition of cytoplasmic microtubule depolymerization (7). This inhibits normal spindle formation and causes cell cycle arrest in the G2/M phase (8). Other studies have suggested that the main mechanism by which paclitaxel acts on prostate cancer cells is via Bcl-2 degradation, which reduces the DNA-protective effect of Bc1-2. This results in DNA cleavage into fragments by nucleic acid enzymes, and ultimately leads to apoptosis (9,10).

Zinc is essential for maintaining the integrity of various enzymes and transcription factors (11). Zinc also serves an important role in gene stability and expression (12). In patients with prostate cancer, zinc concentrations have been demonstrated to be reduced by 60-70% in blood serum and cancerous prostate tissue, and to decrease further as cancer progresses (13,14). Another study reported that the main mechanism by which paclitaxel acts on prostate cancer cells is via Bcl-2 degradation, which reduces the DNA-protective effect of Bc1-2. This results in DNA cleavage into fragments by nucleic acid enzymes, and ultimately leads to apoptosis (9,10).

Under androgen stimulation, the peripheral zone of the prostate exhibits high zinc concentrations (18). In prostate cancer, epithelial cells lose the ability to absorb high concentrations of zinc, and tumor growth increases zinc consumption,
thus resulting in decreased zinc content (19,20). Previous studies have suggested that physiological concentrations of zinc can directly induce prostate cancer cell apoptosis via the mitochondrial pathway, release of mitochondrial cytochrome c and activation of the apoptosis-associated protease caspase-3, which triggers programmed cell death (9,21). Previously, researchers have injected zinc into prostate tumors and found that it inhibited cell growth and prolonged patient survival. Furthermore, treatment with paclitaxel alone has been demonstrated to be associated with the development of drug resistance (22,23). We hypothesized that reduced zinc concentrations may be associated with chemotheraphy resistance in prostate cancer. In the present study, it was assessed whether zinc increased chemosensitivity of PC-3 cells to paclitaxel.

Materials and methods

Cell cultivation. PC-3 cells were obtained from the Prostate Diseases Prevention and Treatment Research Center (Jilin University, Changchun, China) and were cultured in Iscove's modified Dulbecco's medium (IMDM; HyClone; GE Healthcare Life Sciences, Little Chalfont, UK) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Little Chalfont, UK) to prevent nonspecific binding, then with a Ki-67 primary antibody (dilution, 1:1,000; cat. no. 27309-1-AP; ProteinTech Group, Inc. Chicago, IL, USA) at 37°C for 30 min. Following incubation with a goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (dilution, 1:1,000; cat. no., SA00001-2; ProteinTech Group, Inc. Chicago, IL, USA) at room temperature for 40 min, the cells were stained with Hoechst 33342 (1 µg/ml; Sangon Biotech Co., Ltd., Shanghai, China) for 2 min at room temperature, then mounted using aqueous mounting media (glycerin and water, 9:1). Cell staining was detected by fluorescence microscopy at x1,200, magnification.

Flow cytometric analysis of cell apoptosis. Following exposure of PC-3 cells to different treatment conditions (control group; 250 µmol/l zinc group; 10 nmol/l; paclitaxel group, and 250 µmol/l zinc + 10 nmol/l paclitaxel group) for 48 h, they were washed twice with PBS, then collected using EDTA-free trypsin. The cells were collected by centrifugation at 1,000 x g for 5 min at 4°C. Annexin V-FITC (5 µl; Beckman Coulter Inc., Brea, CA, USA) was added and the cells were incubated in the dark for 15 min at 4°C, then 10 µl of propidium iodide (PI; Beckman Coulter Inc.) was added and the cells were incubated in the dark for a further 5 min at 4°C. An Epics-XL-MCL flow cytometer (Beckman Coulter Inc., Brea, CA, USA) was used to detect the rate of cell apoptosis within 1 h of staining.

Flow cytometric analysis of mitochondrial membrane potential. Around 2.7x10^5 cells/well were cultured in 6-well plates. The cells were collected for flow cytometric analysis by washing twice with PBS, followed by treatment with 0.25% EDTA-free trypsin and centrifugation at 900 x g for 5 min at 4°C. Serum-free IMDM (1 ml) and JC-1 (1 µl; Beckman Coulter Inc.) were added and the cells were incubated in the dark at 37°C for 20 min, then centrifuged at 1,500-2,000 x g for 5 min. The culture medium was discarded and the cells were washed twice with PBS, and centrifuged at 2,000 x g for 5 min. Finally, the cells were resuspended in 400 µl IMDM. Mitochondrial membrane potential was analyzed using an Epics-XL-MCL flow cytometer.
Isopropanol (cat. no., A507048; Sangon Biotech Co., Ltd.) was added to the aqueous phase at room temperature for 5 min. Finally, RNA was washed in 75% ethanol and resuspended in RNase-free water. Reverse transcription was conducted using Promega M-MLV reverse transcriptase (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. PCR was conducted using 2X EasyTaq PCR SuperMix (cat. no. AS111-02; Beijing Transgen Biotech Co., Ltd., Beijing, China). The primer sequences and thermocycling conditions are presented in Tables I and II. The images were captured using the Tanon, 1600R image processing system (Tanon Science and Technology Co., Ltd., Shanghai, China). The gray ratio between the target genes and GAPDH was analyzed to measure the expression level of target genes by image processing system (Tanon, 1600R; Tanon Science and Technology Co., Ltd.).

**Western blotting.** PC-3 cells were lysed by incubation with 100-150 µl of radioimmunoprecipitation assay buffer supplemented with phenylmethylsulfonyl fluoride (Roche Diagnostics, Basel, Switzerland), then sonicated 3-5 times for 3-4 sec using ultrasound pyrolysis apparatus (Tomy Seiko Co., Ltd., Tokyo, Japan). Subsequently, the cells were centrifuged at 12,000 x g, for 20 min at 4°C and the protein concentration was measured using Bradford reagent (BioRad Laboratories, CA, USA). The lysates were subjected to 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMB Millipore, Billerica, MA, USA). The membranes were washed with TSB-Tween (Sangon Biotech Co., Ltd.) on the horizontal rocking bed for 5 min, and then blocked with 5% skimmed milk (Sangon Biotech Co., Ltd.) on the horizontal rocking bed for 1 h at room temperature. Bcl-2-associated X, apoptosis regulator (Bax), Bel-2, caspase-3, caspase-9, cleaved caspase-3 and cleaved caspase-9 were detected by western blotting. Bax (dilution, 1:1,000; cat. no., 23931-1-AP) and Bcl-2 (dilution, 1:1,000; cat. no. 12789-1-AP) primary antibodies were purchased from ProteinTech Group, Inc. (Chicago, IL, USA). Cleaved caspase-3 (dilution, 1:1,000; cat. no., 9664), cleaved caspase-9 (dilution, 1:1,000; cat. no., 9509), caspase-3 (dilution, 1:1,000; cat. no., 9662), caspase-9 (dilution, 1:1,000; cat. no., 9504) and actin (dilution, 1:1,000; cat. no., 8456) primary antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). All the primary antibodies were incubated at room temperature for 4 h prior to washing with TBS-Tween. Next, membranes were incubated with a goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (dilution, 1:1,000; cat. no., 9664), cleaved caspase-9 (dilution, 1:1,000; cat. no., 9509), caspase-3 (dilution, 1:1,000; cat. no., 9662), caspase-9 (dilution, 1:1,000; cat. no., 9504) and actin (dilution, 1:1,000; cat. no., 8456) primary antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). All the primary antibodies were incubated at room temperature for 4 h prior to washing with TBS-Tween. Next, membranes were incubated with a goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (dilution, 1:1,000; cat. no., SA00001-2; ProteinTech Group, Inc.) at room temperature for 1 h. Proteins were detected using an enhanced chemiluminescence kit (cat. no. 120702-74; Advansa, Inc., Menio Park, CA, USA). The Syngene Bio Imaging system (GeneGnome HR, Synoptics Ltd., Cambridge, UK) was used to capture the images. And the Image-Pro Plus (Media Cybernetics, Inc., Rockville, MD, USA) was used to quantify the protein expression.

**Statistical analysis.** All data are presented as the mean±standard deviation. SPSS 19.0 (IBM Corp., Armonk, NY, USA) was used to perform one-way analysis of variance and the least significant difference test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.
Results

Zinc increases the sensitivity of PC-3 cells to paclitaxel. First, an MTT assay was used to determine the optimal concentration combination of zinc and paclitaxel. PC-3 cells were treated with different concentrations of zinc and paclitaxel for 48 h. The results showed that 0-250 µmol/l zinc had no significant effect on PC-3 cells compared with untreated cells (Fig. 1A). However, zinc concentrations >250 µmol/l significantly decreased the activity of PC-3 cells. PC-3-cell viability was reduced by ~25% by 10 nmol/l paclitaxel compared with untreated cells (Fig. 1B). The effect of a 48-h incubation with 10 nmol/l paclitaxel and different concentrations of zinc was investigated. Treatment with 10 nmol/l paclitaxel combined with 250 µmol/l zinc reduced PC-3 cell proliferation by ~50% compared with untreated cells (P<0.05; Fig. 1C). Therefore, 10 nmol/l paclitaxel combined with 250 µmol/l zinc was used in subsequent experiments.

To explore the effect of zinc combined with paclitaxel on the proliferative ability of PC-3 cells, a clone formation assay was performed. The cell clone formation rate was significantly reduced in the cells treated with paclitaxel only or zinc combined with paclitaxel, compared with the control cells and cells treated with zinc only (Fig. 2A). Furthermore, the cell clone formation rate was significantly lower in cells treated with zinc combined with paclitaxel than those treated with paclitaxel only (P<0.05; Fig. 2B). This suggested that zinc combined with paclitaxel significantly reduced the proliferation ability of PC-3 cells.

Hoechst staining was used to analyze apoptosis (Fig. 3). Zinc combined with paclitaxel significantly enhanced the fluorescence intensity, and nuclear fragmentation was evident. This indicated that zinc combined with paclitaxel significantly induced PC-3 cell apoptosis (P<0.05). Annexin V and PI staining and flow cytometry revealed that, compared with control treatment (2.28±3.34%) and treatment with zinc only (4.12±2.52%), treatment with paclitaxel alone (12.48±4.34%) and zinc combined with paclitaxel (36.30±3.68%) both increased the rate of PC-3-cell apoptosis, with the combination treatment having most significant effect (P<0.05; Fig. 4A and B). These results suggested that zinc enhanced the sensitivity of PC-3 prostate cancer cells to paclitaxel chemotherapy.

A decrease in mitochondrial membrane potential is a key feature of early-stage apoptosis. A change in JC-1 fluorescence from red to green reflects a change in cell membrane potential. Flow cytometry was used to assess the effect of zinc combined with paclitaxel on the mitochondrial membrane potential of PC-3 cells (Fig. 5A). Treating cells with zinc combined with paclitaxel significantly decreased the ratio of red to green fluorescence (P<0.05; Fig. 5B). This indicates that PC-3 cells treated with zinc and paclitaxel exhibited significantly reduced mitochondrial function compared with control cells, which may reflect early apoptosis. In summary, zinc combined with paclitaxel promoted apoptosis and inhibited cell proliferation. Hence, zinc increased the chemosensitivity of PC-3 cells to paclitaxel.

Zinc combined with paclitaxel induced the mitochondria-mediated apoptosis pathway. To investigate the mechanism by which zinc improved the sensitivity of PC-3 cells to paclitaxel chemotherapy, the mRNA levels of Bax, Bcl-2, caspase-3 and caspase-9 by RT-PCR (Fig. 6A) and the protein expression levels were analyzed by western blotting (Fig. 7A). There was a significant increase in the mRNA...
and protein levels of cleaved caspase-3 and cleaved caspase-9 in PC-3 cells treated with paclitaxel alone and paclitaxel combined with zinc, compared with control cells or those treated with zinc alone (P<0.05; Figs. 6C and 7B). There was no significant change in protein levels of caspase-3 and caspase-9 (Fig. 7B). There was also a significant decrease in the Bcl-2/Bax ratio (P<0.05; Figs. 6B and 7B). These results indicate that zinc improved the sensitivity of PC-3 cells to chemotherapy, possibly via effects on the mitochondria-mediated apoptosis pathway.
Discussion

Current prostate cancer treatment comprises surgery, endocrine therapy, radiotherapy and chemotherapy. Seeing as prostate cancer is often diagnosed at a late stage, the optimal time for surgery is often missed (8,24), making effective chemotherapy important. Paclitaxel has been approved by the Food and Drug Administration as a first-line treatment for hormone-refractory prostate cancer, and has been reported to be the only current drug that can prolong survival and improve quality of life in patients with tumors of the prostate (4,25). Paclitaxel has been widely used in the treatment of breast, ovarian, head and neck, and lung cancers. However, chemotherapy with a single drug has been associated with the development of drug resistance over time (9). Therefore, research based on chemotherapeutic combination strategies is important.
Functional p53 enhances the sensitivity of prostate cancer cells to chemotherapeutic agents, and loss of p53 function in the PC-3 prostate cancer cell line increases paclitaxel resistance (26). Zinc deficiency is a cause of p53 mutation and inactivation (27). Zinc deficiency in prostate cells is closely associated with the occurrence and development of prostate
cancer (2,28). Zinc is an essential trace element for the function of ~200 enzymes in humans, thus serving an important role in genome stability and processes, including gene expression (9,20). Zinc concentrations have been demonstrated to be decreased by 60-70% in the serum and cancerous prostate tissue of patients with prostate cancer, and to decrease further as the cancer progresses (29). We speculate that the reduction in zinc concentration in prostate cancer may be associated with chemotherapy resistance. Therefore, in the present study, PC-3 prostate cancer cells were treated with zinc to investigate whether zinc could enhance sensitivity to chemotherapy.

Previous research has reported that physiological concentrations of zinc can directly induce apoptosis of prostate cancer cells via the mitochondrial pathway. The main mechanism of this is the release of mitochondrial cytochrome c and the activation of caspase-3, which triggers apoptosis (20,30). In the mitochondria-mediated apoptosis pathway, in the presence of deoxyadenosine triphosphate, cytochrome c binds with and activates apoptotic protease activating factor-1 (Apaf-1), and this complex further combines with procaspase-9 to form an apoptosis complex that activates procaspase-9. Activated caspase-9 activates other caspases, including caspase-3, thus inducing apoptosis (31). The Bcl-2 gene family serves an important role in this pathway. Some death receptor-independent stimuli (including ultraviolet ray irradiation and chemotherapy) activate the Bcl-2 gene family. Bcl-2 regulates the opening and closing of the permeability transition pore (32,33). To study the mechanism by which zinc enhances the chemotherapeutic effectiveness of paclitaxel, the expression of apoptosis-associated proteins was investigated by western blotting. The results indicate that the mechanism may involve a decrease in Bcl-2 protein expression levels, and an increase in Bax, cleaved caspase-3 and cleaved caspase-9 protein expression levels (Fig. 7). It is likely that the mitochondria-mediated apoptosis pathway was activated, causing apoptosis via activation of caspases by a cascade reaction.

Although single-agent chemotherapy can have a therapeutic effect against prostate cancer, patients with prostate cancer patients are prone to chemotherapy resistance. This is possibly due to severe zinc deficiency in prostate cancer tissues. The present study suggests that zinc enhances the sensitivity of the PC-3 prostate cancer cell line to the chemotherapeutic agent, paclitaxel. Combining paclitaxel with zinc enhances therapeutic efficacy, and may have potential for overcoming issues associated with single-agent treatment. The present study provides a basis for novel strategies for tumor combination therapy.

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Availability of data and materials

Some datasets are not available as the experimental group is still performing further studies. However, other data is available from the corresponding author on reasonable request.

Authors’ contributions

YLi, YLiu, LS and PZ participated in the research design. PZ, XT, HG, JL and RG conducted the experiments and contributed to data acquisition and analysis. PZ, JS and YYC performed the data analysis. PZ and YLiu contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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