Sildenafil Induces Cell Cycle Arrest and Apoptosis in Human Colorectal Cancer HT-29 Cells

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Abstract: Phosphodiesterase is an enzyme that degrades the phosphodiester bond in the second messenger molecules cAMP and cGMP, and regulates the localization, duration, and amplitude of cyclic nucleotide signaling within subcellular domains. Sildenafil is a potent and selective inhibitor of the type 5 cGMP-specific phosphodiesterase used clinically to treat erectile dysfunction and pulmonary arterial hypertension. In this study, we examined the effect of sildenafil on human colorectal cancer HT-29 cells. Our data showed that sildenafil induced cell cycle arrest and apoptosis, and cotreatment with a ROS scavenger N-acetyl-L-cysteine partially reversed cell apoptosis caused by sildenafil in human colorectal cancer HT-29 cells. Overall, our study suggests that sildenafil appears to be a promising new treatment option for colorectal cancer.

Keywords: Sildenafil, Colorectal cancer, Cell Cycle, Apoptosis.

1. INTRODUCTION

Sildenafil, an inhibitor of cGMP-specific phosphodiesterase type 5 (PDE5), binds to PDE5 and prevents the biotransformation of second messenger 3', 5'-cGMP to 5'-GMP, thus increasing intracellular cGMP levels. It is clinically used to treat erectile dysfunction and pulmonary arterial hypertension [1, 2]. Until now, several groups have evaluated the effect of sildenafil on multiple types of cancers. Sildenafil can inhibit cell growth and induce caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia cells cells [3]. Sildenafil also suppresses the proliferation and migration of human thyroid cancer TPC-1, BCPAP, and 8505C cells [4]. However, sildenafil potentiates a cGMP-dependent pathway to promote melanoma growth and may elevate melanoma risk [5-11], but do not promote primary tumor growth and metastasis of the androgen independent human prostate cancer cell line PC-3 [12]. Our previous report demonstrated that sildenafil could inhibit the growth of human colorectal cancer HCT116 and SW480 cells *in vitro* and *in vivo* [13]. In this study, we examined the effect of sildenafil on human colorectal cancer HT-29 cells, and found that sildenafil induced cell cycle arrest and apoptosis in human colorectal cancer HT-29 cells.

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2. MATERIAL AND METHODS

2.1. Cell Lines, Cell Culture, and Reagents

Human HT-29 colorectal cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 ng/ml) in a humidified incubator at 37°C with 5% CO2. Sildenafil from Shanghai Tauto Biotechnology were dissolved as a stock solution 10mM in DMSO and stored at -20 °C. N-acetly-L-cysteine (Nac) and dihydroethidium (DHE) were purchased from Sigma-Aldrich. Anti-Cycin A (SC-596), Anti-Cycin D1 (SC-718), Anti-Cyclin E (SC-481), Anti-CDK2 (SC-163), antibodies were from Santa Cruz Biotechnology. Anti-CDK6 (3524-1) antibodies were from Abcam. Anti-PARP (9542) antibodies were from Cell Signaling Technologies. Anti-GAPDH (LK9002T) antibodies were from Tianjin Sungene Biotech.

2.2. Cell Viability Assay

Cells were firstly seeded into a 96-well plate at a density of 5000 cells per well, and incubated with drugs in three parallel wells for 72 h. Then MTT was added to each well at a final concentration of 0.5 mg/ml. After incubation for 4 h, formazan crystals were dissolved in 100 mL of DMSO, and absorbance at 570 nm was measured by plate reader. The concentrations required to inhibit growth by 50% (IC_{50}) were calculated from survival curves using the Bliss method [14, 15].

2.3. Cell Cycle Analysis

Cells were harvested and washed twice with cold phosphate-buffered saline (PBS), then fixed with ice-

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cold 70% ethanol for 30 min at 4 °C. After centrifugation at 200 × g for 10 min, cells were washed twice with PBS and resuspended with 0.5 ml PBS containing PI (50 μg/ml), 0.1% Triton X-100, 0.1% sodium citrate, and DNase-free RNase (100 μg/ml), and detected by FCM after 15 min incubation at room temperature in the dark. Fluorescence was measured at an excitation wavelength of 480 nm through a FL-2filter (585 nm). Data were analyzed using ModFit LT 3.0 software (Becton Dickinson).

2.4. Apoptosis Assay

Cell apoptosis was evaluated with flow cytometry (FCM) assay. Briefly, cells were harvested and washed twice with PBS, stained with Annexin V-FITC and propidium iodide (PI) in the binding buffer, and detected by FACSCalibur FCM (BD, CA, USA) after 15 min incubation at room temperature in the dark. Fluorescence was measured at an excitation wave length of 480 nm through FL-1 (530 nm) and FL-2 filters (585 nm). The early apoptotic cells (Annexin V positive only) and late apoptotic cells (Annexin V and PI positive) were quantified.

2.5. Reactive Oxygen Species (ROS) Assay

Cells were incubated with 10 μM of DHE for 30 min at 37 °C, washed twice with PBS and immediately photographed under fluorescent microscope (Olympus, Japan). For each well, 5 fields were taken randomly.

2.6. Western Blot Analysis

Cells were harvested and washed twice with cold PBS, then resuspended and lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 ng/ml PMSF, 0.03% aprotinin, 1μM sodium orthovanadate) at 4°C for 30 min. Lysates were centrifuged for 10 min at 14,000×g and supernatants were stored at -80 °C as whole cell extracts. Total protein concentrations were determined with Bradford assay. Proteins were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% BSA and incubated with the indicated primary antibodies. Corresponding horseradish peroxidase-conjugated secondary antibodies were used against each primary antibody. Proteins were detected using the chemiluminescent detection reagents and films.

2.7. Statistical Analysis

All results are expressed as mean ± standard deviation (SD). Statistical analysis of the difference between treated and untreated groups was performed with Student's t-test. Values of $P< 0.05$ were considered as significant differences.

3. RESULTS

3.1. Sildenafil Induced Cell Cycle Arrest in Human Colorectal Cancer HT-29 Cells

Our previous report showed that sildenafil inhibited the growth of human colorectal cancer HT-29 cells with the IC_{50} values 190.91μM as detected by MTT assay [13]. To explore whether the growth inhibition of HT-29 cells by sildenafil is due to cell cycle arrest, cell cycle distribution was assessed after sildenafil treatment. HT-29 cells were treated with sildenafil (100, 200, 300 μM) for 24h and 48h, stained with PI and examined by FCM. The cell cycle distribution was analyzed with ModFit LT 3.0 software. As shown in Figure **1A** and **1B**, compared to the control groups, sildenafil time- and dose-dependently enhanced the G1 and subG1 population in HT-29 cells. To investigate the molecular mechanism of cell cycle arrest by sildenafil, the cell cycle related proteins were detected by Western blot. The results showed that after treatment of sildenafil, the protein levels of Cyclin A, Cyclin D1, Cyclin E, CDK2, CDK6 were decreased in the time- and dose-dependent manners in HT-29 cells (Figure **1C**).

3.2. Sildenafil Induced Apoptosis in Human Colorectal Cancer HT-29 Cells

To further determine whether the growth inhibition of HT-29 cells by sildenafil is due to apoptosis, cell apoptosis was assessed after sildenafil treatment.HT-29 cells were treated with the different concentrations of sildenafil (100, 200, 300 μM) for 48hr, stained with Annexin V/PI, and examined by FCM. As shown in Figure **2A** and **2B**, sildenafil dose-dependently induced Annexin V+/PI− (early apoptosis) and Annexin V+/PI+ (late apoptosis) in HT-29 cells. To investigate the molecular mechanism of cell apoptosis by sildenafil, the apoptotic related proteins were detected by Western blot. After treatment with sildenafil, the cleaved PARP, which is the marker of apoptosis, was time- and dose-dependently generated in HT-29 cells (Figure **2C**).

3.3. Inhibition of ROS Partially Blocks Sildenafil-Induced Apoptosis in Human Colorectal Cancer HT-29 Cells

We next assessed the role of ROS in the anticancer effects of sildenafil. As shown in Figure **3A** and **B**,

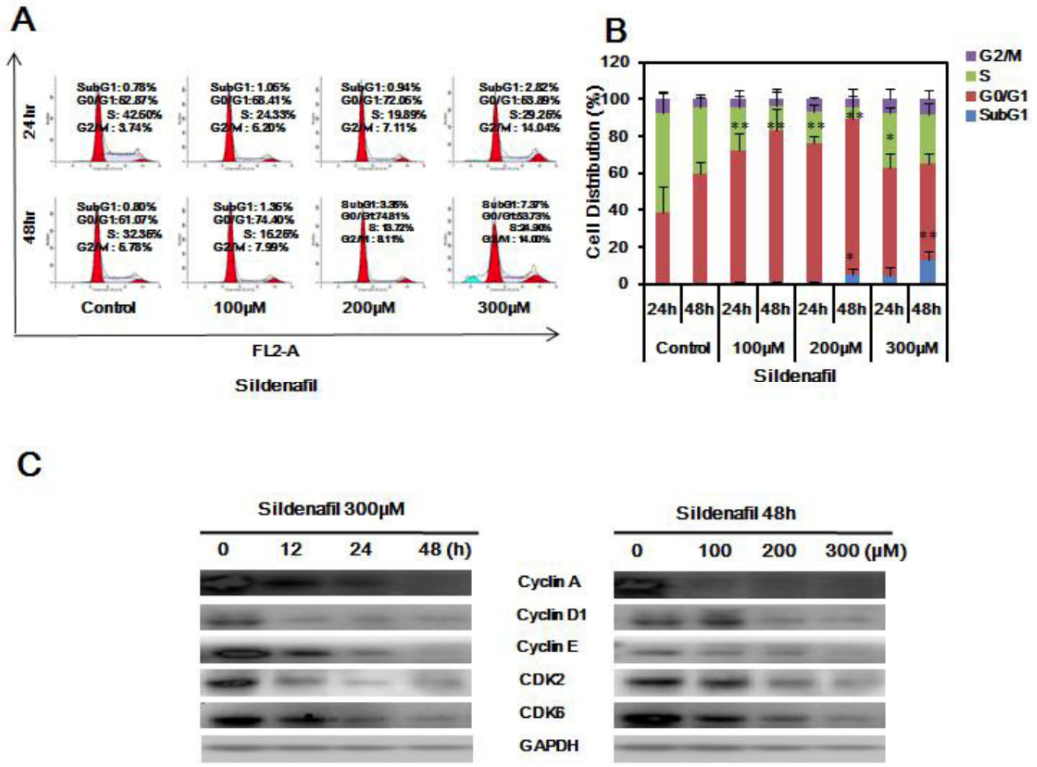

Figure 1: Sildenafil induced cell cyclce arrest in human colorectal cancer HT-29 cells. HT-29 cells were treated with sildenafil at the indicated concentrations and time-points. The distribution of cell cycle was detected by FCM with PI staining. The percentages of subG1, G1/G0, S, G2/M phase were calculated by using ModFit LT 3.0 software. The protein expression was examined by Western blot after lysing cells, and GAPDH was used as loading control. The representative charts (**A**), quantified results (**B**) and Western blot results (**C**) of three independent experiments were shown. **P* < 0.05 and ***P* < 0.01 versus corresponding control.

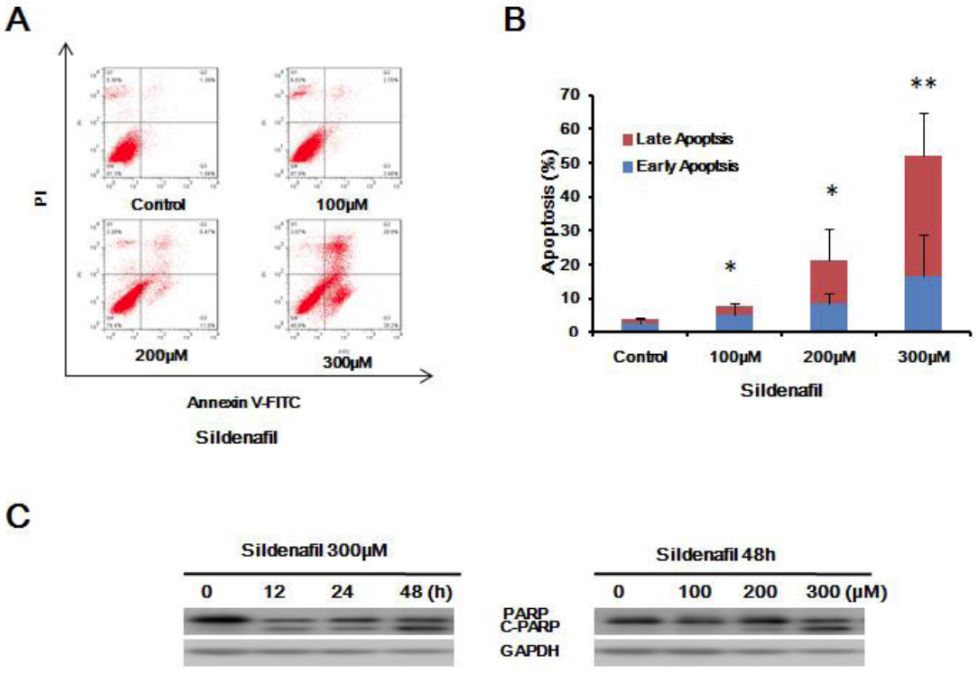

Figure 2: Sildenafil induced apoptosis in human colorectal cancer HT-29 cells. HT-29 cells were treated with sildenafil at the indicated concentrations and time-points. The apoptosis was detected by FCM Annexin V/PI staining. The proportions of Annexin V+/PI- and Annexin V+/PI+ cells indicated the early and late stage of apoptosis. The protein expression was examined by Western blot after lysing cells, and GAPDH was used as loading control. The representative charts (**A**), quantified results (**B**) and Western blot results (**C**) of three independent experiments were shown. **P* < 0.05; ***P* < 0.01 versus corresponding control.

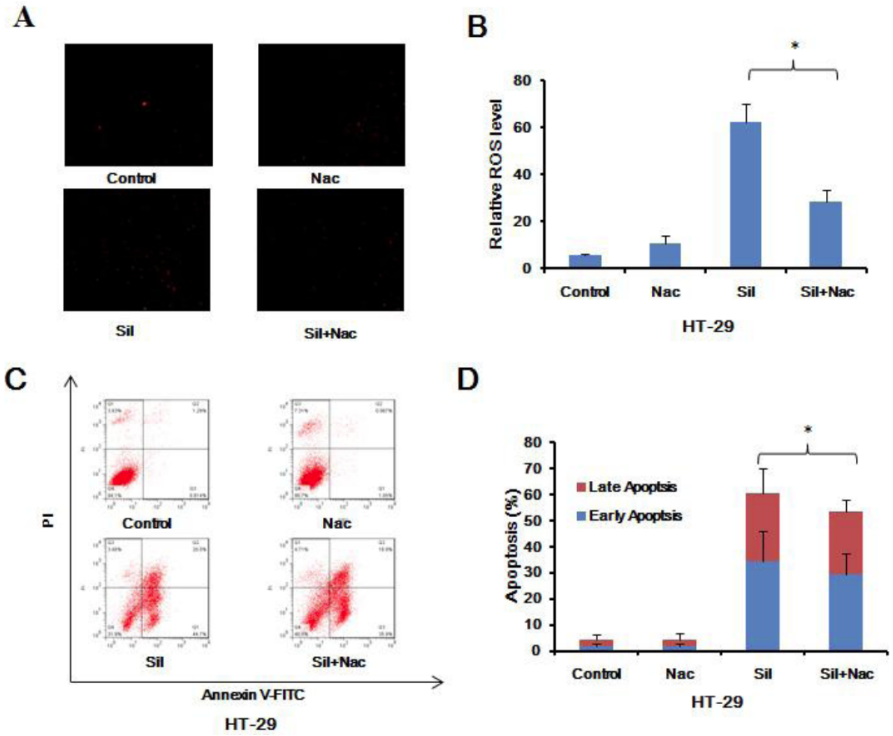

Figure 3: Inhibition of ROS partially blocks sildenafil-induced apoptosis in human colorectal cancer HT-29 cells. HT-29 cells were treated with sildenafil at 300 μM for 48 h in the presence or absence of 5mM Nac pretreatment for 1 h, stained with DHE and photographed under florescent microscope. The representative micrographs (**A**) and quantified results (**B**) of three independent experiments were shown. The apoptosis was detected by FCM with Annexin V/PI staining. The representative charts (**C**) and quantified results (**D**) of three independent experiments were shown. Sil: Sildenafil. *P < 0.05 and **P < 0.01 versus corresponding control.

treatment with sildenafil led to the enhancement of DHE fluorescence intensities in HT-29 cancer cells, and the DHE fluorescent signals induced by sildenafil treatment were reversed by ROS inhibitor Nac in HT-29 cells. Moreover, sildenafil-induced cell apoptosis was partially blocked by Nac in HT-29 cells (Figure **3C** and **3D**).

4. DISCUSSION

In this study, our data showed that sildenafil induced cell cycle arrest and apoptosis in human colorectal cancer HT-29 cells. Sildenafil also increased intracellular ROS levels and Nac partially block sildenafil-induced apoptosis. These results are similar with previous report which demonstrated sildenafil induced apoptosis in B-cell chronic lymphocytic leukemia cells cells [3]. As a chemoadjuvant, sildenafil sensitizes DU145 prostate cancer cells to doxorubicin-mediated apoptosis through CD95 [16]. Furthermore, cotreatment with sildenafil enhanced doxorubicin-induced apoptosis in PC-3 and DU145 prostate cancer cells, which was mediated by enhanced generation of reactive oxygen species [17]. In human breast cancer MCF-7 cells, sildenafil potentiates the antitumor activity of cisplatin by induction of apoptosis and inhibition of proliferation and angiogenesis *in vitro* and *in vivo* [18], and co-delivery of sildenafil and crizotinib promoted a synergistic effect to induce cell apoptosis [19]. Moreover, the synergistic effect of sildenafil with platinum-based agents including cisplatin and carboplatin has been demonstrated to induce cell apoptosis in lung cancer [20]. Sildenafil and curcumin interacted in a greater than additive fashion to kill gastrointestinal tumor cells via endoplasmic reticulum stress and reactive oxygen/ nitrogen species induced apoptosis *in vitro* and *in vivo* [21]. Therefore, sildenafil may be a promising anticancer agent against colorectal cancer, which has supported by recent reports that sildenafil suppresses inflammation-driven colorectal cancer in mice treated with azoxymethane/dextran sulfate sodium [22, 23].

In summary, our data demonstrated that sildenafil induced cell cycle arrest and apoptosis in human colorectal cancer HT-29 cells. Sildenafil appears to be a promising new treatment option for colorectal cancer.

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CONFLICTS OF INTEREST

There is no conflict of interest.

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