Original Article
Sildenafil inhibits the growth of human colorectal cancer *in vitro* and *in vivo*

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Abstract: Colorectal cancer is the third most common human cancer with frequent overexpression of the cGMP-specific phosphodiesterase 5 (PDE5). In the present study, we investigated that the anticancer effect of sildenafil on human colorectal cancer *in vitro* and *in vivo*, which is a potent and selective inhibitor of PDE5 for the treatment of erectile dysfunction and pulmonary arterial hypertension in the clinic. Sildenafil significantly induced cell growth inhibition, cell cycle arrest and apoptosis of human colorectal cancer with increased intracellular reactive oxidative specie (ROS) levels, which were accompanied by obvious alterations of related proteins such as CDKs, Cyclins and PARP etc. Pretreatment with ROS scavenger N-acetyl-L-cysteine could reverse sildenafil-induced ROS accumulation and cell apoptosis. Inhibition of the activity of protein kinase G with KT-5823 could enhance sildenafil-induced apoptosis. Furthermore, sildenafil caused the reduction of xenograft models of human colorectal cancer in nude mice. Overall, these findings suggest that sildenafil has the potential to be used for treatment of human colorectal cancer.

Introduction

Colorectal cancer currently is the third most common human cancer with more than 1 million new cases every year in the world [1]. Treatments for colorectal cancer include surgery, chemotherapy, radiation therapy and immunotherapy [2]. Despite progress in decreasing the death rates of colorectal cancer has been accelerated by improving the diagnosis and combinational treatment, the five-year survival rate for colorectal cancer is less than 60% [3]. Therefore, it is urgent to develop novel therapeutic strategies against colorectal cancer.

Sildenafil (Viagra®), a potent and selective inhibitor of cGMP-specific phosphodiesterase type 5 (PDE5), is used to treat erectile dysfunction (ED) and pulmonary arterial hypertension (PAH) in the clinic. Sildenafil competitively binds to PDE5 with cGMP due to their similar structures, which enhances the levels of cGMP to activate protein kinase G (PKG) resulting in the relaxation of vascular smooth muscle and the increase of blood inflow [4]. PDE5 is widely expressed in the vascular, pulmonary, visceral smooth muscles, platelets, gastrointestinal epithelial cells, and cerebellum, etc [5]. Overexpression of PDE5 is detected in multiple types of cancer [6-9], which has also been confirmed in various cell lines originating from breast cancer (MCF-7, HTB-26, MDA-MB-468), prostate cancer (LNCAP, PC3), bladder cancer (HTB-76, HT1376) and colorectal cancer (HT29, HCT116, SW480, T84) [10]. These findings suggest that PDE5 may play an important role in tumorigenesis and that targeting PDE5 may be a promising anticancer method. Actually, the anticancer effect of sildenafil and other PDE5 inhibitors have been evaluated by several groups. It has

been reported that sildenafil and vardenafil could suppress tumor cell growth and induce caspase-dependent apoptosis of B-cell chronic lymphocytic leukemia cells *in vitro* [11]. The non-specific PDE5 inhibitor exisulind and its analogues selectively induced the apoptosis of various human prostate, colon and breast cancer cells, which was owing to their inhibitory effects on PDE5 expression, thereby enhancing the cGMP-induced activation of PKG [12-21]. In the present study, we investigated that anticancer effect of sildenafil in human colorectal cancer *in vitro* and *in vivo*.

Material and methods

Cell lines, cell culture, and reagents

Human colorectal cancer cell lines (HT-29, SW480, SW620, HCT116, SW1116) 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% CO_2. Sildenafil from ApexBio technology 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-PARP (9542) and Anti-β-Catenin (9582) antibodies were from Cell Signaling Technologies. Anti-CDK2 (SC-163), Anti-CDK4 (SC-260), Anti-Cycin A (SC-596), Anti-Cycin D1 (SC-718), Anti-Cyclin E (SC-481) and Anti-Hsp90 (SC-13119) antibodies were from Santa Cruz Biotechnology. Anti-CDK6 (3524-1) antibodies were from Abcam. Anti-GAPDH (LK9002T) antibodies were from Tianjin Sungene Biotech.

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 μl 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 [22, 23].

Cell cycle analysis

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

fixed with ice-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) [24, 25].

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 FACS Calibur 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 [26, 27].
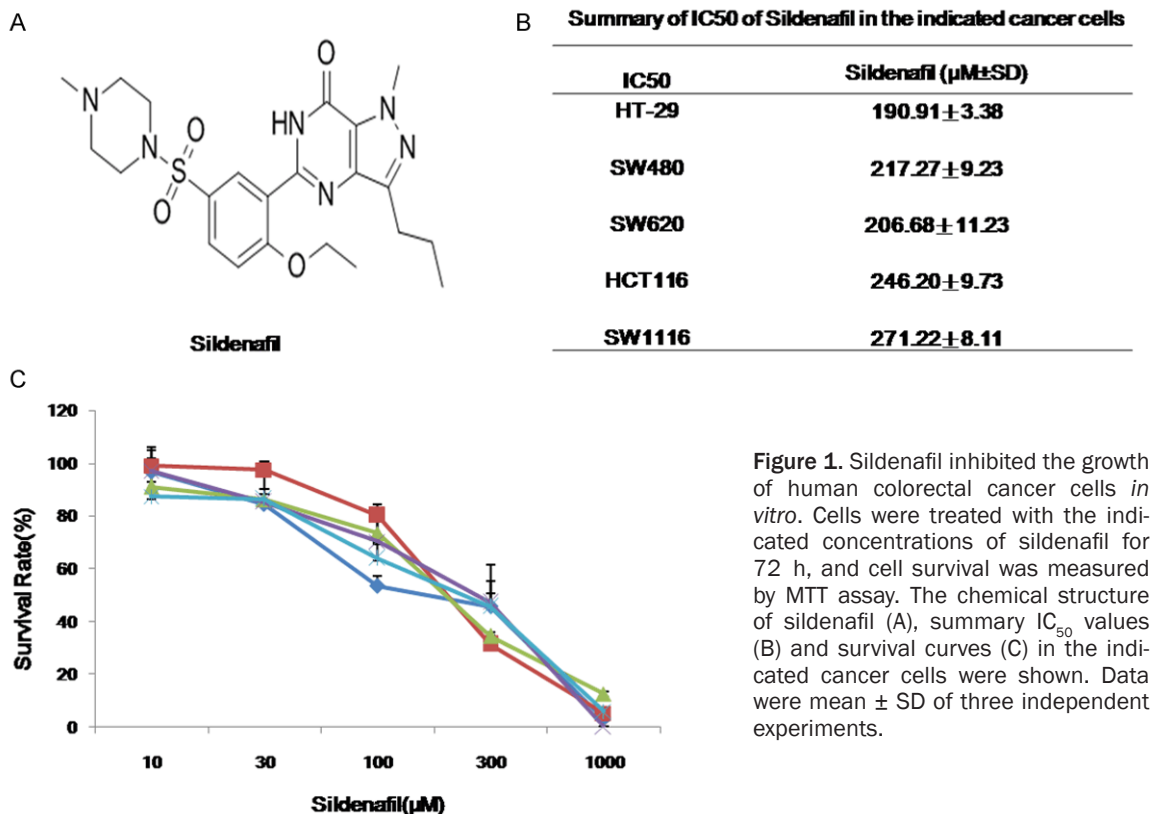
Reactive oxygen species (ROS) assay

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 [28, 29].

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 chemi-

A

Sildenafil

B

Summary of IC50 of Sildenafil in the indicated cancer cells	
IC50	Sildenafil (µM±SD)
HT-29	190.91±3.38
SW480	217.27±9.23
SW620	206.68±11.23
HCT116	246.20±9.73
SW1116	271.22±8.11

C

Figure 1. Sildenafil inhibited the growth of human colorectal cancer cells *in vitro*. Cells were treated with the indicated concentrations of sildenafil for 72 h, and cell survival was measured by MTT assay. The chemical structure of sildenafil (A), summary IC_{50} values (B) and survival curves (C) in the indicated cancer cells were shown. Data were mean ± SD of three independent experiments.

luminescent detection reagents and films [30, 31].

Nude mice xenograft tumor assay

Balb/c nude mice were obtained from the Guangdong Medical Laboratory Animal Center and maintained with sterilized food and water. Six female nude mice with 5 weeks old and 20 g weight were used for each group. Each mouse was injected subcutaneously with SW480 or HCT116 cells (3×10^6 in 200 µl of medium) under the shoulder. When the subcutaneous tumors were approximately 0.4×0.4 cm^2 (two perpendicular diameters) in size, mice were randomized into three groups, and were taken orally with vehicle alone (0.9% saline), sildenafil (50 mg/kg) and sildenafil (150 mg/kg) every two days. The body weights of mice and the two perpendicular diameters (A and B) of tumors were recorded. The tumor volume (V) was calculated according to the formula:

$$v = \frac{\pi}{6}(\frac{A + B}{2})^3$$

The mice were anaesthetized after experiment, and tumor tissue was excised from the mice and weighted. The rate of inhibition (IR) was calculated according to the formula [32, 33]:

$$IR = 1 - \frac{\text{Mean tumor weight of experimental group}}{\text{Mean tumor weight of control group}} \times 100\%$$

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.

Results

Sildenafil inhibited the growth of human colorectal cancer cells in vitro

To evaluate the effect of sildenafil on colorectal cancer cells, cell survival was detected by MTT assay. Five human colorectal cancer cell lines HT-29, SW480, SW620, HCT116 and SW1116 were treated with increasing concentrations of sildenafil range from 10 µM to 1 mM for 72 h. As shown in **Figure 1**, the results showed that the growth of five human cancer cell lines were similarly inhibited by sildenafil in a concentra-

A

SW480 Sildenafil

SW480 Sildenafil

B
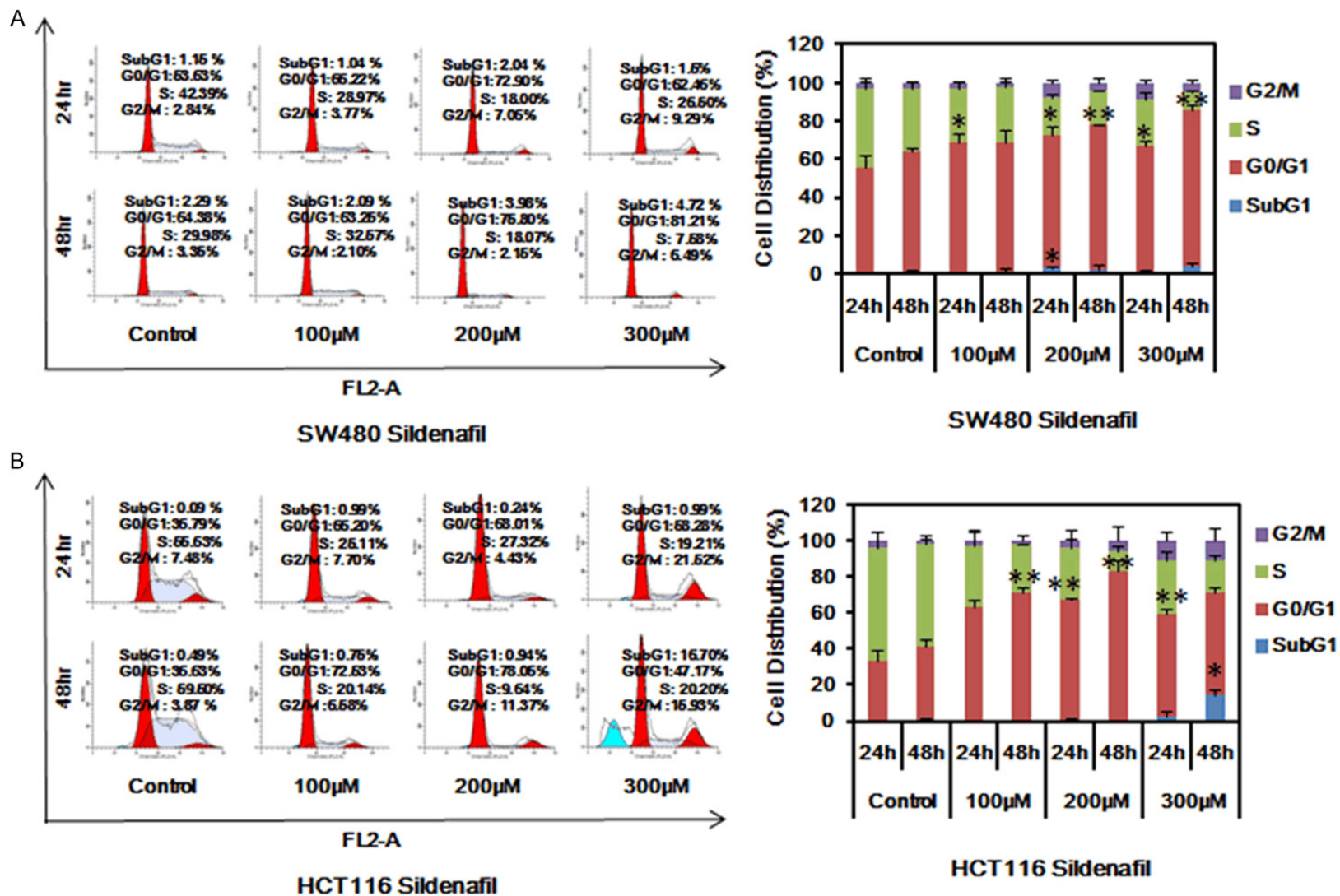
HCT116 Sildenafil
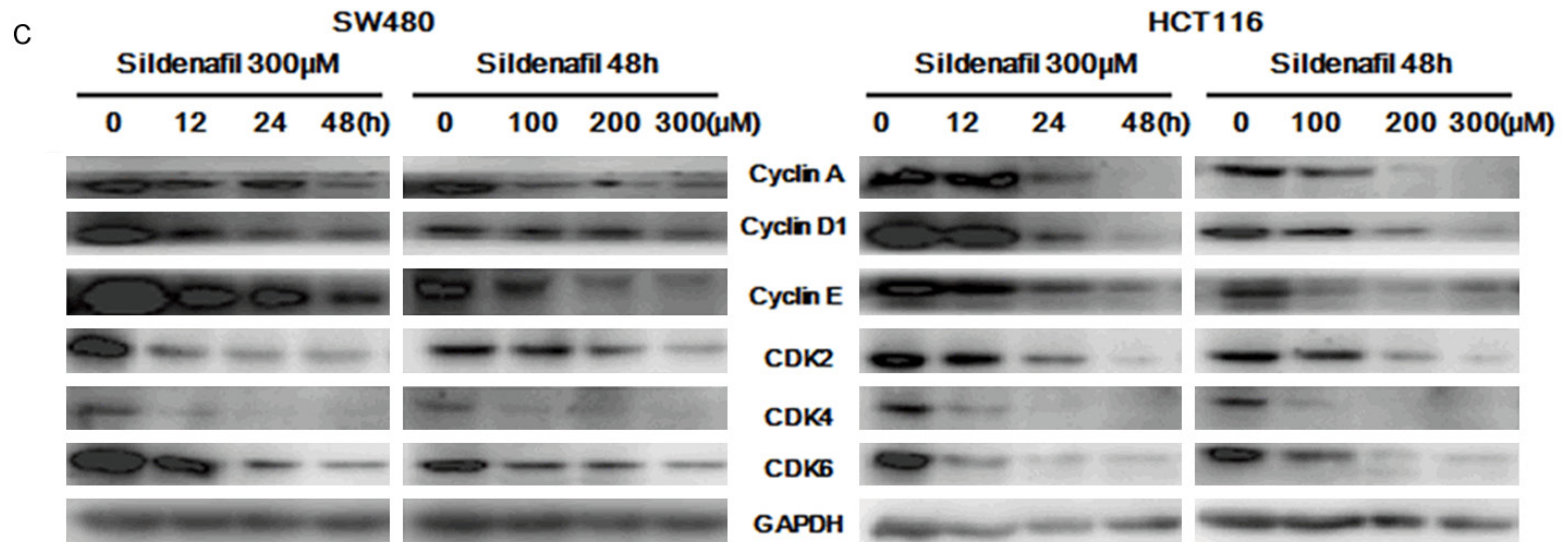
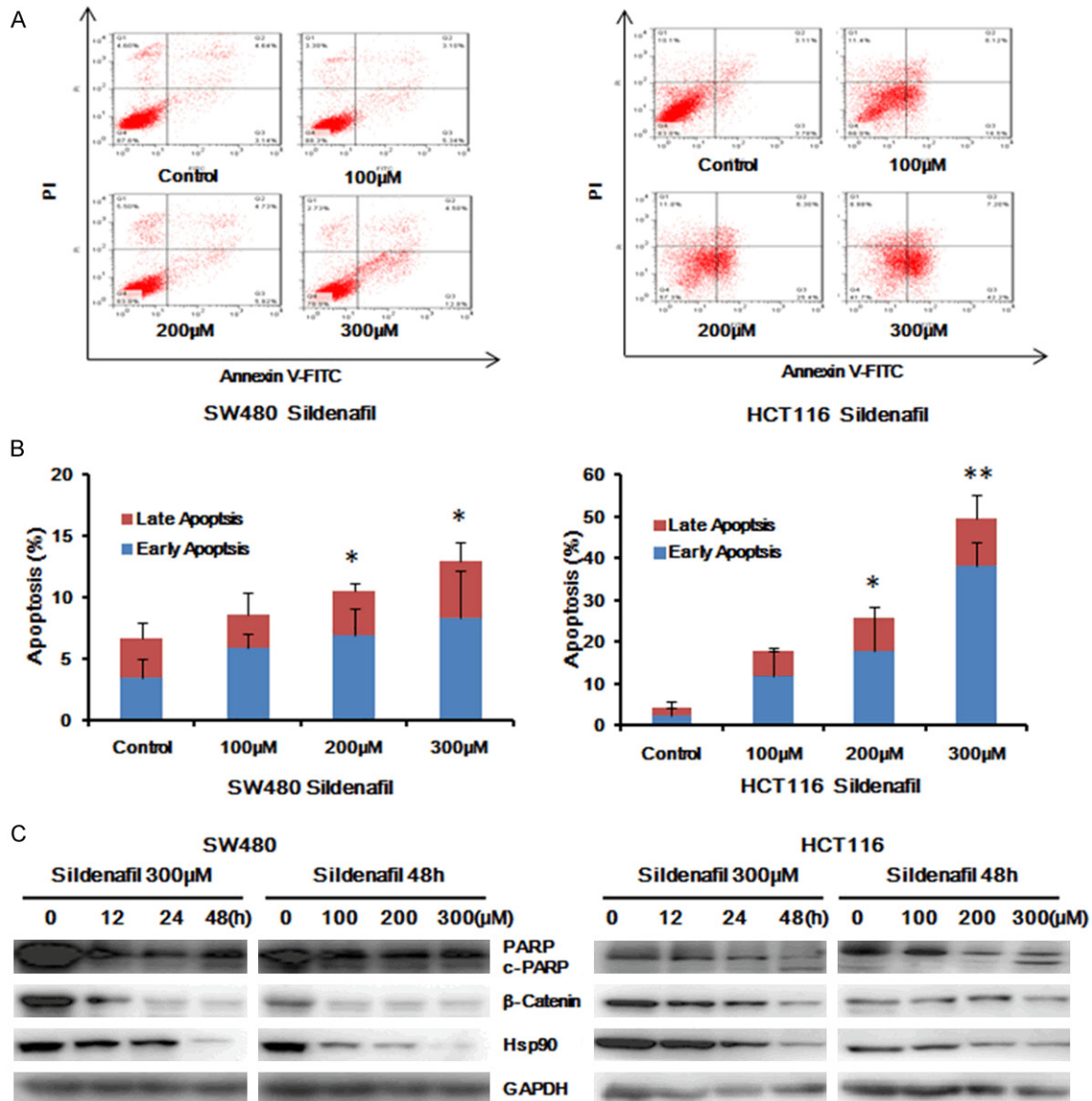HCT116 Sildenafil

Figure 2. Sildenafil induced G1 cell cycle arrest in human colorectal cancer cells. SW480 (A) and HCT116 (B) 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, G0/G1, S, G2/M phase were calculated by using ModFit LT 3.0 software. The protein expression was examined by Western blot, and GAPDH was used as loading control. The representative charts, quantified results and Western blot results (C) of three independent experiments were shown. *$P < 0.05$ and **$P < 0.01$ versus corresponding control.

Figure 3. Sildenafil induced apoptosis in human colorectal cancer cells. SW480 (A) and HCT116 (B) 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, and GAPDH was used as loading control. The representative charts, quantified results and Western blot results (C) of three independent experiments were shown. *P < 0.05 and **P < 0.01 versus corresponding control.

tion-dependent manner with the IC$_{50}$ values range from 190 to 271 μM.

Sildenafil induced G1 cell cycle arrest in human colorectal cancer cells

To explore whether the growth inhibition of colorectal cancer cells by sildenafil is due to cell cycle arrest, cell cycle distribution was assessed after sildenafil treatment. Colorectal cancer cells SW480 and HCT116 were treated with sildenafil (100, 200, 300 μM) for 24 h and 48 h, stained with PI and examined by FCM. The cell cycle distribution was analyzed with ModFit LT 3.0 software. As shown in **Figure 2A** and **2B**, compared with the control groups, sildenafil induced the accumulation of G0/G1 phase in SW480 and HCT116 cells. To further investigate the molecular mechanism of cell cycle arrest by sildenafil, the cell cycle related
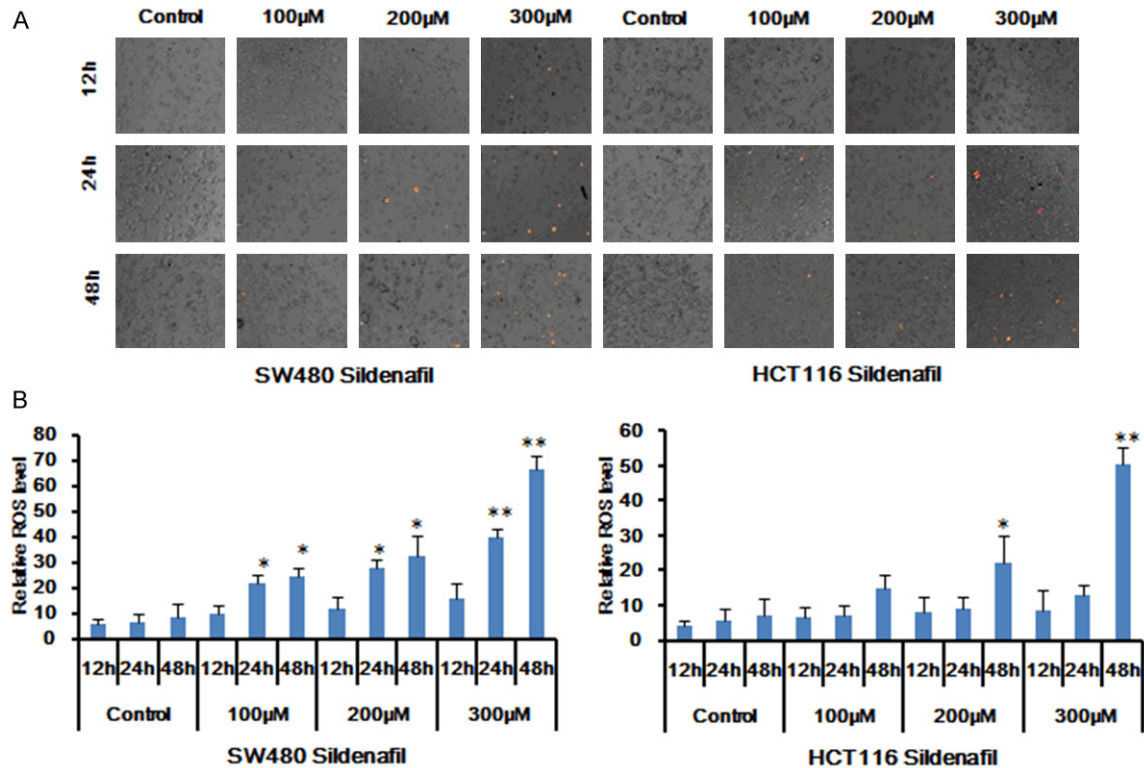
Figure 4. Sildenafil induced ROS accumulation in human colorectal cancer cells. SW480 and HCT116 cells were treated with sildenafil at the indicated concentrations and time-points, stained with DHE and photographed under microscope. The representative micrographs (A) and quantified results (B) of three independent experiments were shown. *$P < 0.05$ and **$P < 0.01$ versus corresponding control.

proteins were detected by Western blot. The results showed that the protein levels of Cyclin A, Cyclin D1, Cyclin E, CDK2, CDK4 and CDK6 were down-regulated in the time- and dose-dependent manners in SW480 and HCT116 cells after sildenafil treatment (**Figure 2C**).

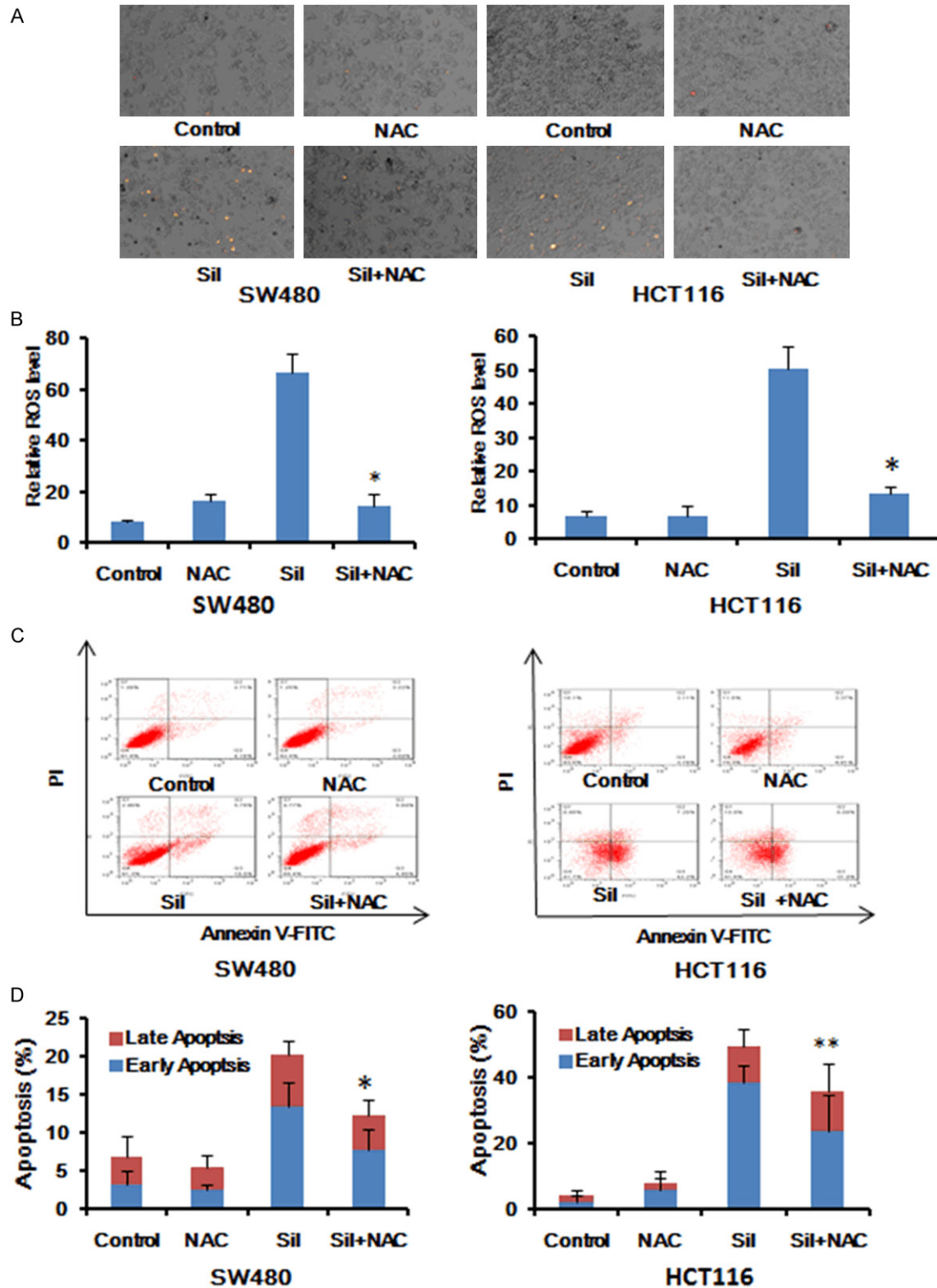*Sildenafil induced apoptosis in human colorectal cancer cells*

In addition to the evaluation of sildenafil induced growth inhibition and cell cycle arrest, its effects on apoptosis were analyzed by FCM. Both SW480 and HCT116 cells were treated with the different concentrations of sildenafil (100, 200, 300 μM) for 48 h, stained with Annexin V/PI, and examined by FCM. As shown in **Figure 3A** and **3B**, sildenafil treatment significantly induced apoptosis in both SW480 and HCT116 cells, and both proportions of Annexin V+/PI- (early stage of apoptosis) and Annexin V+/PI+ (late stage of apoptosis) cells were increased with the elevated sildenafil concentrations. To further investigate the molecular mechanism of cell apoptosis by sildenafil, the

apoptotic related proteins were detected by Western blot. After treatment with sildenafil, the protein levels of full-length PARP, β-Catenin and Hsp90 were time- and dose-dependent decreased in both SW480 and HCT116 cells (**Figure 3C**).

ROS generation was critical for sildenafil-induced apoptosis in human colorectal cancer cells

Many chemotherapeutic agents induce the death of cancer cells through elevating the level of intracellular ROS [34]. To explore the role of ROS in the anticancer effect of sildenafil in colorectal cancer cells, we used dihydro-ethidium (DHE) as ROS fluorescent probe, which can be oxidized by ROS to oxide ethidium that binds with DNA to emit the detectable red fluorescence [29]. As shown in **Figure 4A** and **4B**, sildenafil exposure resulted in a time- and concentration-dependent ROS accumulation in both SW480 and HCT116 cells. To further explore the relationship between sildenafil induced apoptosis and ROS generation,
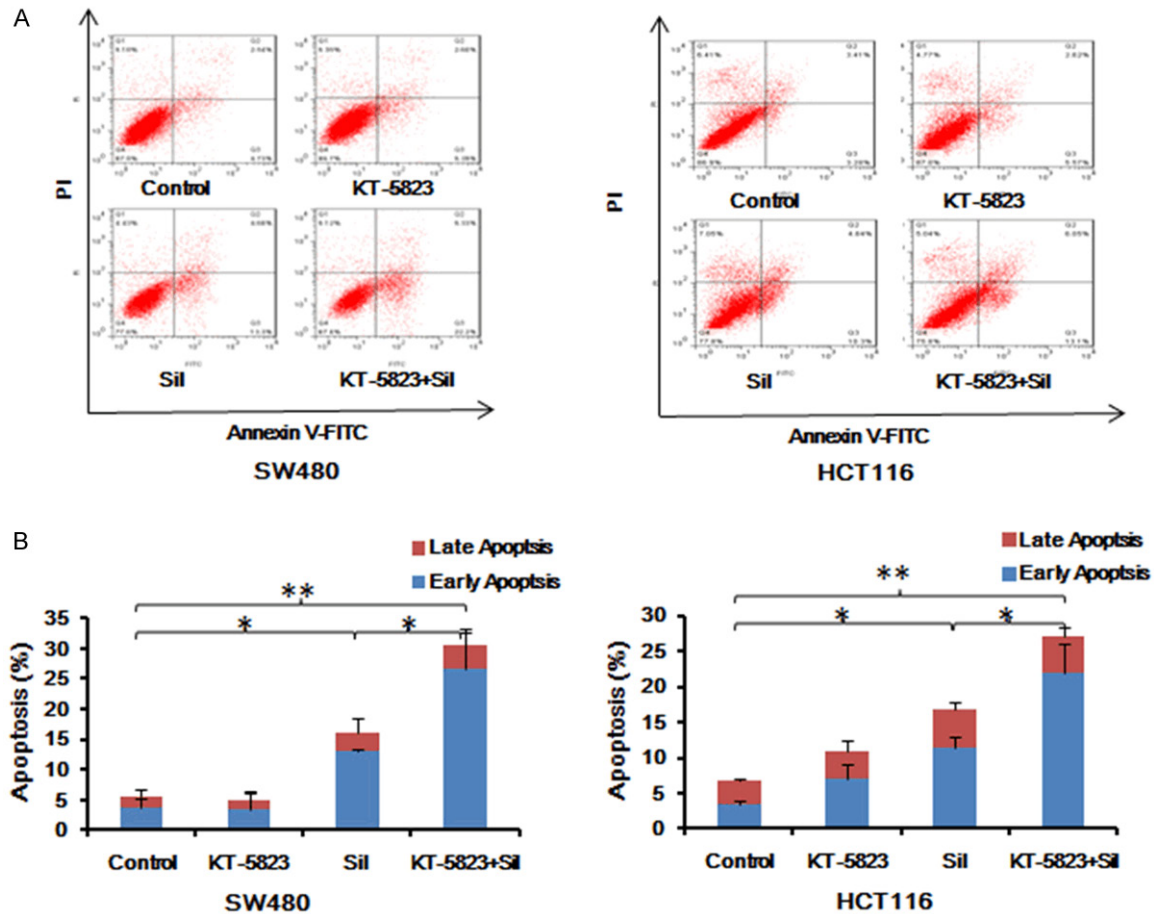
Figure 5. ROS generation was critical for sildenafil-induced apoptosis in human colorectal cancer cells. SW480 and HCT116 cells were treated with sildenafil at 300 µM for 48 h in the presence or absence of 5 mM NAC pretreatment for 1 h, stained with DHE and photographed under microscope. The representative 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.

Figure 6. Inhibition of PKG enhanced sildenafil-induced apoptosis in human colorectal cancer cells. SW480 and HCT116 cells were treated with sildenafil at 100 μM and KT-5823 at 2 μM alone or combination for 48 h. The apoptosis was detected by FCM with Annexin V/PI staining. The representative charts (A) and quantified results (B) of three independent experiments were shown. Sil: Sildenafil. *P < 0.05 and **P < 0.01 versus corresponding control.

both cells were treated with sildenafil for 48 h in the presence or absence of the anti-oxidative agent NAC pretreatment for 1 h and stained with DHE. As predicted, the sildenafil-induced ROS accumulation was greatly reduced by NAC due to its ability to elevate the intracellular glutathione to prevent the production of ROS (**Figure 5A** and **5B**). Moreover, cell apoptosis was detected by FCM with Annexin V/PI staining. The sildenafil-induced apoptosis were partially blocked by NAC in both SW480 and HCT116 cells (**Figure 5C** and **5D**). Taken together, these data suggested that ROS is critical for sildenafil-induced apoptosis in colorectal cancer cells.

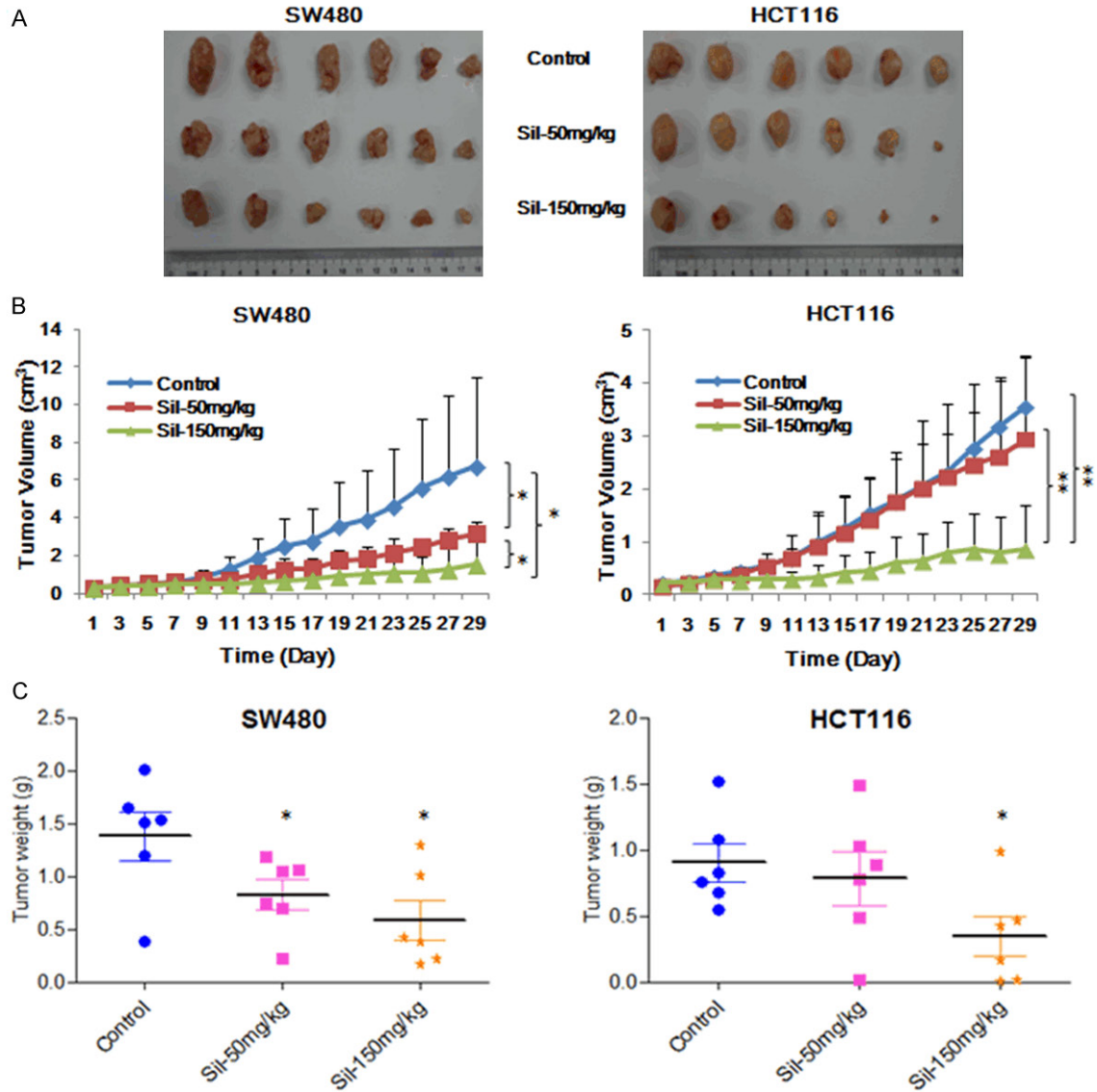*Inhibition of PKG enhanced sildenafil-induced apoptosis in human colorectal cancer cells*

PDE5 inhibitor exerts their biological effects mainly through the PKG pathway [35]. To evalu-

ate the function of PKG on sildenafil-induced apoptosis in colorectal cancer cells, both SW480 and HCT116 cells were treated with sildenafil at 100 μM and a specific PKG inhibitor KT-5823 at the nontoxic concentration of 2 μM for 48 h, stained with Annexin V/PI, and examined by FCM. As shown in **Figure 6A** and **6B**, KT-5823 alone did not significantly induce apoptosis, but obviously promoted sildenafil-induced apoptosis in both SW480 and HCT116 cells, suggesting inhibition of PKG could enhance sildenafil-induced apoptosis in colorectal cancer cells.

Sildenafil inhibited the subcutaneous xenograft growth of human colorectal cancer in nude mice

To investigate the anticancer effect of sildenafil on colorectal cancer *in vivo*, the nude mice with subcutaneous xenografts of colorectal cancer

Figure 7. Sildenafil inhibited the subcutaneous xenograft growth of human colorectal cancer in nude mice. Each nude mouse was injected subcutaneously with SW480 or HCT116 cells (3 × 10⁶ in 200 µl of medium) under the shoulder. When the subcutaneous tumors were approximately 0.4 × 0.4 cm² (two perpendicular diameters) in size, the nude mice were randomly divided into three groups (n = 6 mice/group), and taken orally with vehicle alone

(0.9% saline), sildenafil (50 mg/kg), sildenafil (150 mg/kg) every two days. The body weights of mice and tumor volume were recorded. The mice were anaesthetized after experiment, and tumor tissue was excised from the mice and weighted. The original tumors (A), tumor volumes (B), tumor weights (C) and summary data (D) were shown. Sil: Sildenafil. *P < 0.05 and **P < 0.01 versus corresponding control.

cells SW480 and HCT116 were treated with two doses of sildenafil. As shown in **Figure 7A-C**, compared with control group, sildenafil clearly inhibited the tumors growth by reducing the volume and weight of SW480 and HCT116 xenografts. The inhibition rates of sildenafil at the doses of 50 and 150 mg/kg were 40.1% and 57.8% in SW480 xenografts as well as 13.3% and 61.4% in HCT116 xenografts, respectively (**Figure 7D**). In addition, the weight of mice were equal between before and after experiments in each group, suggesting treatment of sildenafil at the indicated doses did not cause significant side effects in mice (**Figure 7D**).

Disscussion

Recently, the anticancer effect of sildenafil has been evaluated by several groups. Sildenafil could directly trigger caspase-dependent apoptosis of B-chronic lymphocytic leukemia cells *in vitro* [11], and enhance endogenous antitumor immunity by inhibiting the function of myeloid-derived suppressor cells [36]. In a rat brain tumor model, sildenafil improved the efficacy of doxorubicin by increasing the permeability of blood-brain tumor barrier [37]. Moreover, sildenafil was reported to increase the chemotherapeutic efficacy of doxorubicin in prostate cancer and breast cancer cells without amplifying its toxicity [38, 39]. Sildenafil could also enhance the effects of other chemotherapeutic agents including mitomycin C, doxorubicin, cisplatin, and gemcitabine in bladder and pancreatic cancer cells [40]. It has been reported that sildenafil could enhance the cytotoxicity of celecoxib in tumor cells including colorectal cancer, hepatoma, glioblastoma and medulloblastoma in an NOS-dependent activation of CD95 induced cell death [41]. Additionally, co-delivery of sildenafil and crizotinib with PEG-PLA micellar carriers showed synergistic and improved anti-tumoral therapy in MCF-7 breast cancer cells [42], and combinatorial delivery of sildenafil-crizotinib-palbociclib using TPGS-PLA micelles could improve cancer treatment in A549 non-small lung cancer cells [43]. Our and others previous data have shown that sildenafil is an inhibitor of ABCB1/P-gp, ABCG2/BCRP,

ABCC4/MRP4, ABCC5/MRP5, and ABCC10/MRP7 [44-48]. However, the *in vivo* studies suggested that sildenafil is not sufficient to cause a meaningful interaction with the drug transporters ABCB1/P-gp and ABCG2/BCRP [49].

In this report, we firstly showed that sildenafil had anti-growth effect on human colorectal cancer cells *in vitro* and *in vivo*. Treatment with sildenafil induced cell arrest at G1 phase and apoptosis with the increasing accumulation of intracellular ROS in the concentration- and time-dependent manners. Sildenafil-induced apoptosis was partially inhibited by ROS inhibitor NAC but enhanced by PKG inhibitor KT-5823. PKG as the downstream kinase of cGMP normally activates specific proteins which control the contractile activity of smooth muscle cells. Interestingly, accumulating evidences indicate that cancer cells express the lower levels of PKG in comparison with normal tissue [50, 51]. The increased activation of PKG by cGMP induced apoptosis and growth arrest in breast cancer cells [52], and the increased expression of PKG decreased invasiveness and tumor growth of human colon cancer xenograft models in nude mice [51]. PDE inhibitor sulindac sulfide specifically inhibited cGMP hydrolysis and activated PKG to produce apoptosis in breast cancer cells, but had no effect on normal human mammary epithelial cells [12]. However, inhibition of cGMP-induced PKG activation by PKG inhibitor KT-5823 also suppressed the growth of breast cancer cells through induction of cell apoptosis [52]. This is consistent with our results that inhibition of PKG could enhance sildenafil-induced apoptosis. It is possible that the anticancer action of PDE5 inhibitors including sildenafil may be mainly attributed to cGMP/PKG regulated downstream pathways. CGMP-activated PKG could decrease the expression of Wnt/β-catenin [18] and cyclin D1 [53] to induce caspase-dependent apoptosis and cell growth arrest, which was also detected in our study.

In summary, our data demonstrated that sildenafil significantly inhibited the growth of human colorectal cancer cells *in vitro* and *in vivo* by

inducing cell cycle arrest and apoptosis with the increasing intracellular ROS levels. Sildenafil-induced apoptosis was partially reversed by inhibition of ROS generation but enhanced by inhibition of PKG activity. These results suggest that sildenafil may be a promising anticancer agent against colorectal cancer.

Disclosure of conflict of interest

None.

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