

Ginsenoside Rg3 suppresses the proliferation of prostate cancer cell line PC3 through ROS-induced cell cycle arrest

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Abstract. To investigate the potential antitumor effects of ginsenoside Rg3 in prostate cancer cells, the androgen-insensitive prostate cancer cell line PC3 was cultured and incubated with ginsenoside Rg3 *in vitro*. Cell number counts, cell proliferation assays and senescence-associated β -galactosidase (SA- β -gal) staining were performed to evaluate cell proliferation. The results demonstrated that ginsenoside Rg3 led to cell proliferation arrest; ginsenoside Rg3 decreased the number of cells and increased the positive SA- β -gal staining rate in PC3 cells. Cell cycle analysis by flow cytometry revealed that ginsenoside Rg3 interfered with the G1/S transition in PC3 cells. The mechanism involved in ginsenoside Rg3-induced cell proliferation arrest was then further investigated. This indicated that the level of reactive oxygen species (ROS) in PC3 cells was upregulated by ginsenoside Rg3 treatment. Furthermore, pretreatment with N-acetyl-L-cysteine, a scavenger of ROS, was able to reverse the effects on cell number and cell cycle arrest induced by ginsenoside Rg3 in PC3 cells. These results indicate that ginsenoside Rg3 exhibits anticancer effects on prostate cancer cells through ROS-mediated arrest of the cell cycle.

Introduction

Ginseng has been used as a traditional medical herb for thousands of years in Asian countries (1). Ginsenoside Rg3, one of the active ingredients extracted from ginseng, has demonstrated potential anticancer activity in multiple malignancy types (2). Previous research indicates that ginsenoside Rg3 is able to induce cell apoptosis (3,4), attenuate cell migration and invasion (5-7), and enhance the sensitivity of cancer cells to chemotherapy (8,9). Similar research has been performed in prostate cancer, a common malignancy in elderly males (10). In such studies, ginsenoside Rg3 was identified to attenuate cell migration by inhibiting the expression of aquaporin 1 (11) and to enhance the antitumor effects of docetaxel in prostate cancer cells (12). At a high dosage (250 μ M), ginsenoside Rg3 has also been revealed to induce cell apoptosis in the prostate cancer cell line LNCaP (13). However, to the best of our knowledge, the exact role of ginsenoside Rg3 and the molecular mechanism involved in its effects on prostate cancer cells remains to be fully understood.

Reactive oxygen species (ROS) consist of reactive oxygen ions and peroxides. ROS are produced in normal metabolic processes and at high concentrations they can cause detrimental effects on biomolecules, including nucleic acids, proteins and lipids (14). In several cancer cell lines, ginsenoside Rg3 exerts its anticancer activity by modulating the intracellular ROS level. However, the regulatory effects of ginsenoside Rg3 on ROS levels are not consistent among different cancer cell types. The accumulation of ROS induced by ginsenoside Rg3 has been observed in hepatoma, breast cancer, glioblastoma, leukemia and Jurkat cells, and has been identified to contribute to cancer cell apoptosis (15-18). However, one study has demonstrated that in Lewis lung carcinoma cells, ginsenoside Rg3 induces tumor cell apoptosis by reducing intracellular ROS (3). These previous studies indicate that ginsenoside Rg3 exhibits various effects on different types of cancer cells through the modulation of ROS. However, to the best of our knowledge, few studies have investigated the role of ginsenoside Rg3 in modulating the levels of ROS in prostate cancer cells.

The present study identified that ginsenoside Rg3 significantly increases the number of intracellular ROS in a

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dose-dependent manner and subsequently induces cell cycle arrest but not apoptosis in the prostate cancer cell line PC3.

Materials and methods

Cells and reagents. PC3, a prostate cancer cell line, was obtained from the German Cancer Research Center (Heidelberg, Germany) and cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), at 37°C and 5% CO₂.

Drugs. Ginsenoside Rg3 (Tianjin YiFang S&T Co., Ltd., Tianjin, China) was dissolved in dimethylsulfoxide (DMSO) at 100 mM. N-acetyl-L-cysteine (NAC; Beyotime Institute of Biotechnology, Haimen, China) was dissolved at 1 M in PBS.

Senescence-associated β -galactosidase (SA- β -gal) staining. PC3 cells were cultured in six-well plates at a density of 5×10^5 cells/well and treated with DMSO or 50 μ M ginsenoside Rg3 for 48 h at 37°C in an atmosphere of 5% CO₂, followed by SA- β -gal staining using Senescence β -Galactosidase Staining kit (Beyotime Institute of Biotechnology). Images were captured using a phase-contrast microscope (magnification, x200). In total, six fields of view were randomly selected in each well and the percentage of cells stained positive was calculated. The data are expressed as the mean \pm standard deviation (SD).

Cell count. PC3 cells were digested with 0.5% trypsin (YuanYe S&T, Shanghai, China) and suspended in RPMI-1640 medium. Cell counts were performed using a hemocytometer and a light microscope (magnification, x100). All data are expressed as the mean of triplicates \pm SD.

Cell proliferation assays. PC3 cells were seeded in 24-well plates at a density of 5×10^4 cells/well and on the following day they were treated with DMSO or ginsenoside Rg3 (25, 50 or 100 μ M) for 72 h at 37°C in an atmosphere of 5% CO₂. Cell Counting Kit-8 (CCK8) solution (US Everbright, Inc., San Ramon, CA, USA) was added to each well, followed by incubation at 37°C for 2 h, according to the manufacturer's protocol. The absorbance of each well was measured at 450 nm. All results are expressed as the mean of triplicates \pm SD.

Cell cycle analysis. PC3 cells were treated with DMSO or 50 μ M ginsenoside Rg3 for 48 h at 37°C in an atmosphere of 5% CO₂. A cell cycle and apoptosis analysis kit (Beyotime Institute of Biotechnology) was used and flow cytometry assays were performed as described previously (19).

ROS assays. PC3 cells were plated in 24-well plates at a density of 5×10^4 cells/well and treated with DMSO or ginsenoside Rg3 (25, 50 or 100 μ M) at 37°C for 72 h in an atmosphere of 5% CO₂. 2,7-Dichlorodihydrofluorescein diacetate stain (DCFH-DA) was diluted to 10 μ M in serum-free RPMI-1640 medium and was used to treat PC3 cells for 20 min at 37°C. Subsequently, cells were washed with serum-free RPMI-1640 medium three times. An ROS assay kit (Beyotime Institute

of Biotechnology) was used to evaluate ROS levels according to the manufacturer's protocol. Images were captured using a fluorescence microscope (magnification, x100).

NAC treatment. PC3 cells were cultured in 24-well plates at a density of 5×10^4 cells/well. The following day, the cells were precultured with 10 mM NAC for 2 h, followed by treatment with DMSO or 50 μ M ginsenoside Rg3 for a further 0, 24, 48 and 96 h at 37°C in an atmosphere of 5% CO₂. Cell counts were performed with a hemocytometer and a light microscope (magnification, x100). All data are expressed as the mean of triplicates \pm SD.

Statistical analysis. All statistical analysis was performed using SPSS software (version 23.0; IBM Corp., Armonk, NY, USA). Data are presented as the mean \pm SD. Analysis of variance (ANOVA) was performed to analyze the data. One-way ANOVA was used for complete random designed data and repeated measures ANOVA was used for repeated measured designed data. A Fisher's least significant difference test was used to perform multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Ginsenoside Rg3 inhibits PC3 cell proliferation in vitro. PC3 prostate cancer cells were seeded in six-well plates and treated with DMSO or 50 μ M ginsenoside Rg3 for 48 h. SA- β -gal staining revealed that ginsenoside Rg3 significantly increased the percentage of positively stained cells (Fig. 1A). The cells were then cultured in 24-well plates at 5×10^4 cells/well, followed by treatment with DMSO or 50 μ M ginsenoside Rg3 for 72 h. The number of cells in each well was counted and cell proliferation was evaluated using a CCK8 assay. The results demonstrated that ginsenoside Rg3 exhibited significant inhibitory effects on cell proliferation *in vitro* compared with DMSO (Fig. 1B and C).

Ginsenoside Rg3 inhibits the G₁/S transition in the PC3 cell cycle. To further study the molecular mechanism involved in the inhibition of cell proliferation by ginsenoside Rg3, flow cytometry analysis was performed to examine the cell cycle of PC3 cells treated with DMSO or 50 μ M ginsenoside Rg3 for 48 h. Ginsenoside Rg3 significantly induced cell cycle arrest in the G₀/G₁ phase and significantly decreased the percentage of cells in the S phase (Fig. 2). These results indicate that treatment with ginsenoside Rg3 inhibits cell cycle transition from the G₁ phase to the S phase in PC3 cells. However, apoptosis of PC3 cells induced by ginsenoside Rg3 was not observed in the current study according to the results of flow cytometry assays (data not shown).

Ginsenoside Rg3 increases ROS levels in PC3 cells in a dose-dependent manner. Oxidative stress acts as a pivotal modulator in the proliferation and apoptosis of cancer cells, and an imbalance in the production and scavenging of ROS triggers the progression of cancer (20). In the current study, different doses of ginsenoside Rg3 (0, 25, 50 and 100 μ M) were used to treat PC3 cells cultured in 24-well plates at 5×10^4 cells/well for 72 h. Compared with the control group, cell counting and CCK8

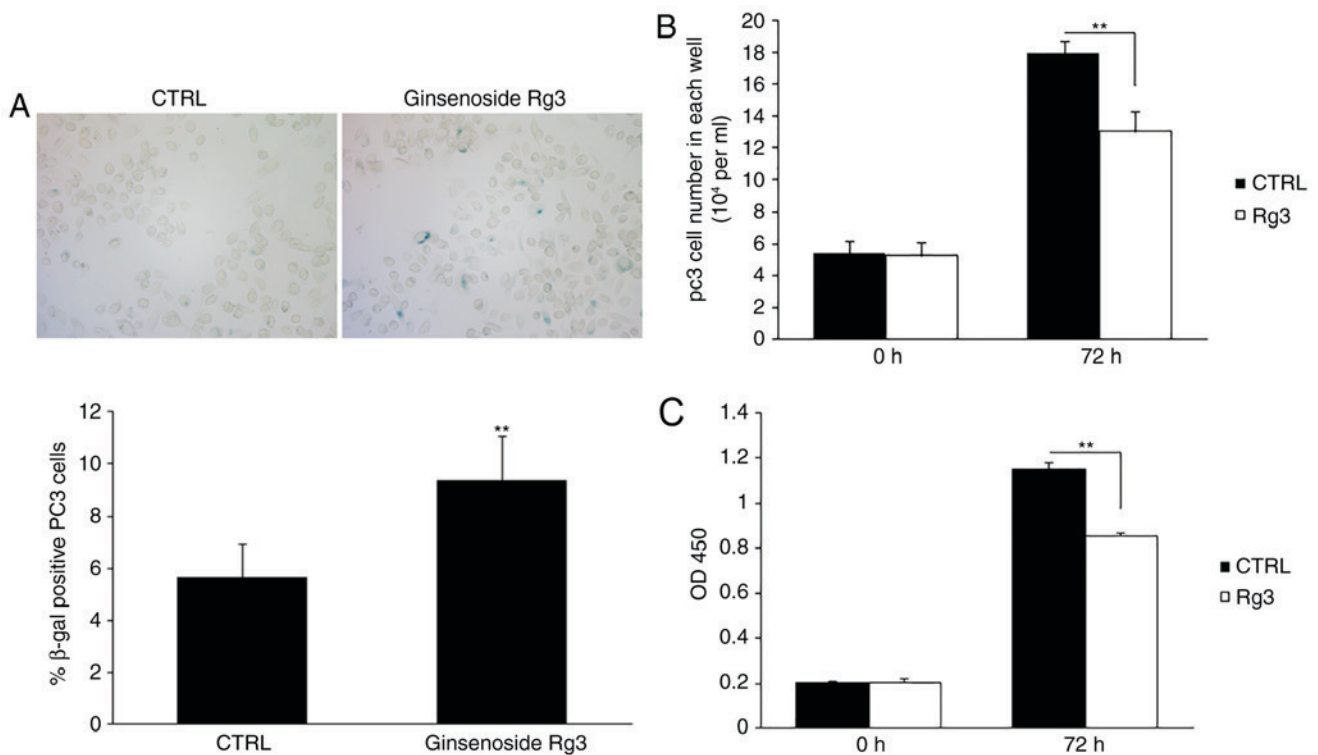


Figure 1. Ginsenoside Rg3 inhibits the proliferation of PC3 cells. (A) PC3 cells were treated with DMSO or 50 μ M ginsenoside Rg3 for 48 h. SA- β -gal staining was performed and the percentage of positively stained cells was calculated. Magnification, x200. ** P <0.01 vs. CTRL. (B) PC3 cells were treated with DMSO or 50 μ M ginsenoside Rg3 for 72 h, followed by Cell Counting Kit-8 assays. ** P <0.01. All data were obtained from three independent experiments and are presented as the mean \pm standard deviation. DMSO, dimethylsulfoxide; CTRL, control; β -gal, β -galactosidase; Rg3, ginsenoside Rg3; OD, optical density.

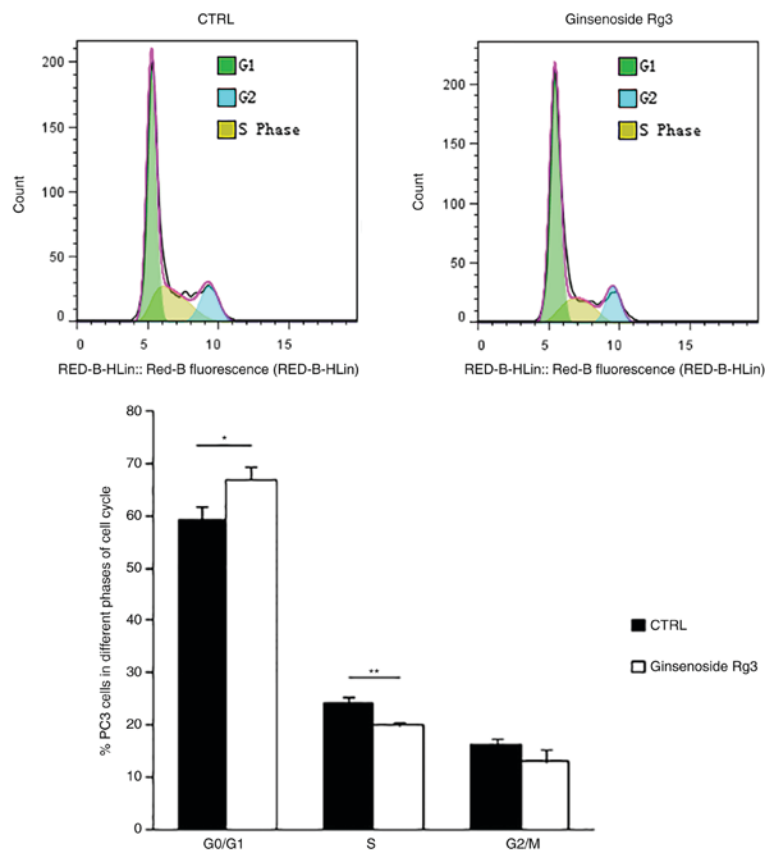


Figure 2. Ginsenoside Rg3 induces cell cycle arrest in PC3 cells. PC3 cells were treated with dimethylsulfoxide or 50 μ M ginsenoside Rg3 for 48 h, followed by flow cytometry analysis. All data were obtained from three independent experiments and are presented as the mean \pm standard deviation. * P <0.05, ** P <0.01. CTRL, control.

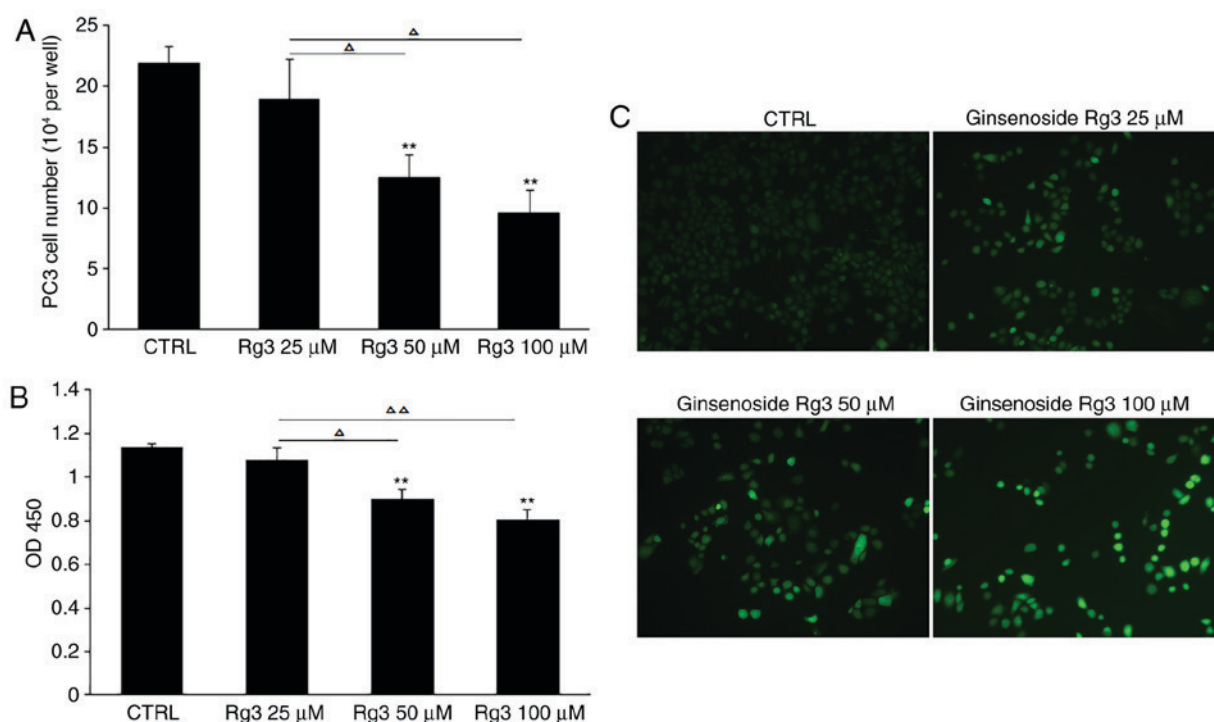


Figure 3. Ginsenoside Rg3 inhibits cell proliferation and induces the accumulation of ROS in PC3 cells in a dose-dependent manner. (A) PC3 cells were treated with various doses of ginsenoside Rg3 (0, 25, 50 and 100 μ M) for 72 h, followed by cell counting. (B) Cell proliferation was measured by Cell Counting Kit-8 assay. (C) 2,7-Dichlorodihydrofluorescein diacetate staining was performed to evaluate the level of ROS. Images were captured using a fluorescence microscope. Magnification, $\times 100$. All data were obtained from three independent experiments and are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. CTRL, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$. ROS, reactive oxygen species; Rg3, ginsenoside Rg3; CTRL, control; OD, optical density; CTRL, control.

analysis demonstrated that 50 and 100 μ M ginsenoside Rg3 significantly inhibited cell proliferation. In addition, compared with 25 μ M ginsenoside Rg3 treatment, 50 and 100 μ M ginsenoside Rg3 exhibited significant inhibitory effects on PC3 cell proliferation (Fig. 3A and B). In addition, DCFH-DA staining was performed to evaluate ROS levels and an accumulation of intracellular ROS was observed in PC3 cells, suggesting a potential association between ginsenoside Rg3-induced cell cycle arrest and increased levels of ROS (Fig. 3C).

Elimination of intracellular ROS with NAC can block ginsenoside Rg3-induced cell cycle arrest in PC3 cells. To investigate the effect of intracellular ROS accumulation on the arrest of cell proliferation induced by ginsenoside Rg3, PC3 cells were precultured with 10 mM NAC for 2 h, followed by treatment with DMSO or 50 μ M ginsenoside Rg3 for a further 0, 24, 48 and 96 h. Cell counting revealed that the elimination of intracellular ROS by NAC significantly blocked the ginsenoside Rg3-induced proliferation inhibition in PC3 cells (Fig. 4A). Flow cytometry analysis was also performed 48 h following treatment with DMSO or ginsenoside Rg3 in PC3 cells. Pretreatment with NAC decreased the cell cycle arrest caused by ginsenoside Rg3 and reestablished the transition of PC3 cells from the G₁ phase to the S phase. The results indicated that compared with the control group, ginsenoside Rg3 significantly increased the proportion of cells in the G₀/G₁ phase and decreased the proportion of cells in the S phase. However, no significant differences were identified in the proportion of cells in the G₀/G₁ phase or the S phase when treated with NAC or NAC+Rg3 compared with the control (Fig. 4B).

Discussion

Oxidative stress is a pivotal factor associated with the pathology of prostate cancer (20). A previous study has identified that the accumulation of ROS induced by a redox disorder can contribute to carcinogenesis resulting from epigenetic regulation and macromolecular damage, including DNA instability (21). Therefore, certain antioxidants are assumed to have therapeutic value in the treatment of prostate cancer (22). The Selenium and Vitamin E Cancer Prevention Trial study was performed with a large sample size and long duration to evaluate the benefits of selenium and vitamin E supplementation in the treatment of prostate cancer, however, the study failed to identify any benefits (23). Therefore, previous studies suggest that antioxidant therapy in cancer treatment requires further assessment.

Antioxidants may provide a feasible strategy for cancer treatment in the early stage of disease, however, they may not provide a feasible strategy in the later stages, particularly in cases involving high levels of ROS that are below the toxic threshold. At the later stages of disease, decreased levels of ROS may have no effects on the progression of cancer or may even exhibit procarcinogenic effects (24).

The current study identified that increased levels of ROS inhibited the proliferation of PC3 cells. However, using a different prostate cancer cell line, DU145, it was revealed that ginsenoside Rg3 increased the intracellular level of ROS but enhanced cell viability, instead of inducing cell proliferation arrest or cell death (data not shown). This result may partially be due to a distinct tolerance of ROS in the two cell lines.

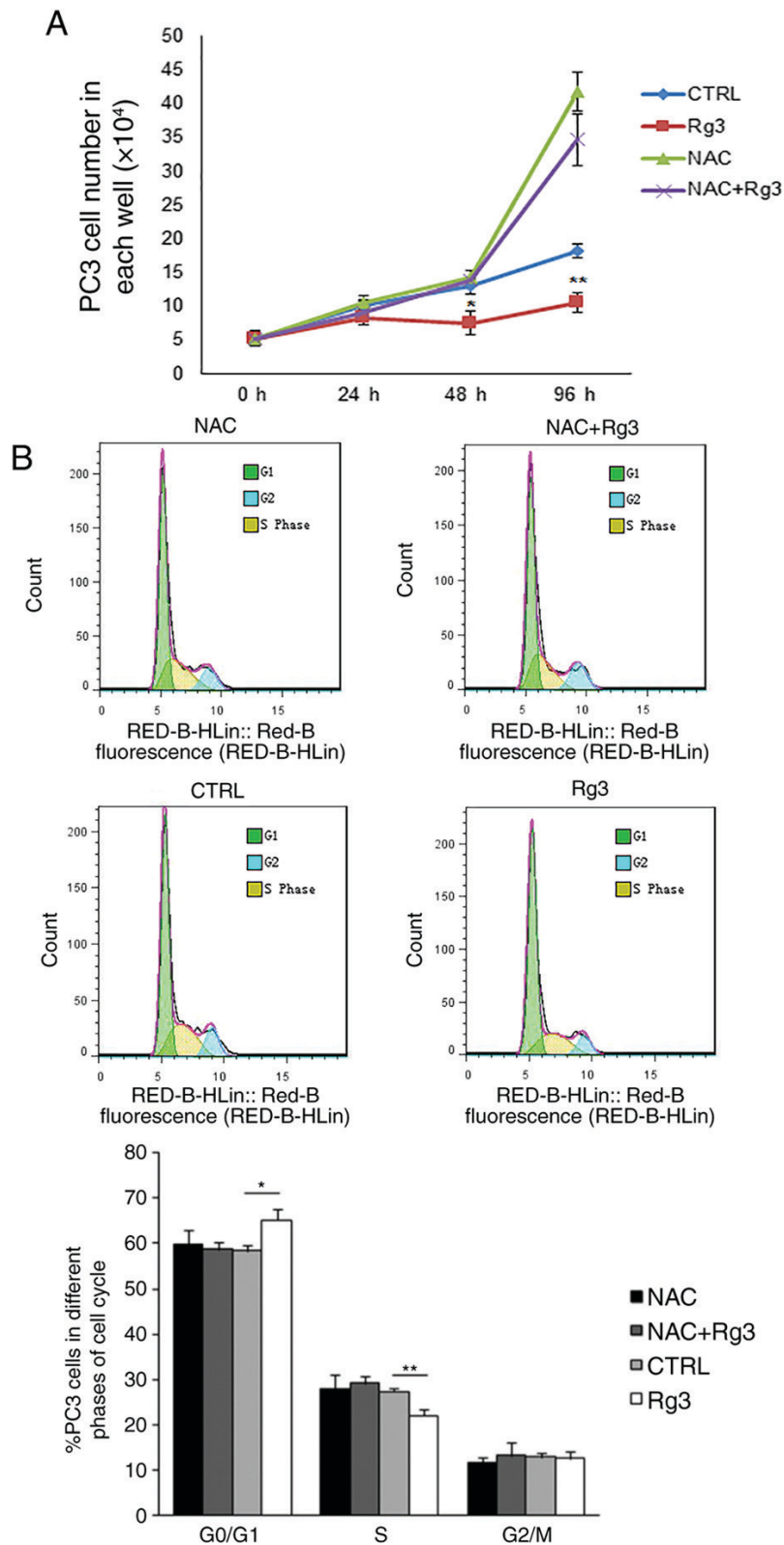


Figure 4. NAC reverses ginsenoside Rg3-inhibited PC3 cell proliferation. (A) PC3 cells were precultured with 10 mM NAC for 2 h and then treated with 50 μ M ginsenoside Rg3 for 0, 24, 48 and 96 h, followed by cell counting. Cell number was significantly decreased with ginsenoside Rg3 treatment at 48 and 96 h compared with the control. However, no significant differences of cell number were observed between the NAC and NAC+Rg3 groups at each time point. * P <0.05, ** P <0.01 vs. CTRL. (B) PC3 cells were precultured with 10 mM NAC for 2 h and then treated with 50 μ M ginsenoside Rg3 for 48 h. Flow cytometry analysis was performed to investigate the cell cycle. * P <0.05; ** P <0.01. All data were obtained from three independent experiments and are presented as the mean \pm standard deviation. NAC, N-acetyl-L-cysteine; CTRL, control; Rg3, ginsenoside Rg3.

A previous study compared the responses of PC3 cells and DU145 cells to ionizing radiation; this revealed that DU145

cells exhibit a higher resistance to radiation due to lower basal levels of ROS, a higher glutathione (GSH) content and

a higher GSH/glutathione disulfide ratio, suggesting DU145 cells possess a greater tolerance to radiation-induced ROS compared with PC3 cells (25). A recent study also identified that phosphatase and tensin homolog (PTEN)-deficient cancer cells with upregulated Akt exhibit high intracellular ROS levels and are more sensitive to ROS-induced cell death compared with PTEN wild-type cell lines (26). This may partially explain the different responses of the PTEN wild-type cell line DU145 and the PTEN-deficient cell line PC3 to ginsenoside Rg3-induced ROS accumulation (27). Therefore, ginsenoside Rg3 should be used with caution in the treatment of prostate cancer as the drug may serve either a pro-cancer or anticancer role depending on the subtype of the disease.

In the current study, ginsenoside Rg3 increased the levels of ROS in prostate cancer cells in a dose-dependent manner. A previous study has demonstrated that ginsenoside Rg3 downregulates ROS levels and inhibits cellular senescence in prostatic stromal cells (28). This suggests that ginsenoside Rg3 may conversely regulate ROS levels in prostate cancer cells and stromal cells. The molecular mechanism involved requires further investigation and the complex effects of ginsenoside Rg3 on both cancer cell types and the tumor microenvironment should be carefully considered before this agent is utilized in cancer treatment.

In summary, the current study revealed that ginsenoside Rg3 induces intracellular ROS accumulation in the prostate cancer cell line PC3 and subsequently inhibits cell proliferation via ROS-induced cell cycle arrest.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

FZ and YP conceived and designed the experiments. YP, RZ, XY and NK performed the experiments. HY, LB, YS and ZZ analyzed the data. HY and ZZ wrote the manuscript. All authors have 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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