

Original Research Article

Ginsenoside induces cell death in breast cancer cells via ROS/PI3K/Akt signaling pathway

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Abstract

Purpose: To study the influence of ginsenoside on breast carcinoma, and the mechanism of action involved.

Methods: Different concentrations of ginsenoside were used to treat MCF-7 breast cancer cell line. Cell viability was measured by MTT assay, while protein expressions of p-Akt and p-PI3K were determined using Western blotting. The concentrations of reactive oxidative reactants and reactive oxygen species (ROS) were assessed using fluorescence immunoassay and immunofluorescence assay. The mechanism of action involved in ginsenoside-mediated apoptosis was determined based on ROS/PI3K/Akt signaling pathway.

Results: There was no change in the inhibition of MCF-7 cell proliferation in control cells with time ($p > 0.05$). However, inhibition of MCF-7 cell proliferation in ginsenoside group was significantly higher than that in the control group ($p < 0.05$); furthermore, it increased with time and ginsenoside concentration. Apoptosis was markedly and concentration-dependently higher in ginsenoside-treated MCF-7 cells than in controls ($p > 0.05$). There were lower protein levels of p-PI3K and p-Akt in ginsenoside-exposed MCF-7 cells than in control group; the protein expressions decreased with increase in ginsenoside concentration ($p < 0.05$). The expressions of ROS in ginsenoside-treated MCF-7 cells declined, relative to the untreated group; in addition, the expressions decreased with increase in ginsenoside concentration ($p < 0.05$).

Conclusion: Ginsenoside suppresses proliferation of MCF-7 cell line, and exerts apoptotic effect on the cells via inhibition of the ROS/PI3K/Akt signal pathway. This provides a new approach to treat breast cancer.

Keywords: Breast cancer cells, Ginsenoside, Apoptosis, ROS/PI3K/Akt signaling pathway

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INTRODUCTION

Breast carcinoma is associated with mammary gland epithelial cells, and has become a serious threat to women's physical and mental health [1].

The etiology of breast cancer is not completely understood. However, recent studies have found that the incidence of breast cancer has a certain regularity, especially in women with high risk factors for the disease [2]. The common clinical

symptoms of breast cancer are mass/lump in breast, nipple discharge, skin changes, areola abnormalities and axillary lymphadenoma [3].

Saponins, the major bioactive compounds in ginseng, have anti-aging, memory-enhancing, hepatoprotective, anti-tumor and anti-inflammatory effects [4].

Ginsenoside is a saponin that has been reported to be effective against myocardial ischemia reperfusion injury, tumors, hyperglycemia, and liver diseases. Moreover, it exerts anti-inflammatory effects. Although clinical findings have shown that ginseng saponin inhibits liver cancer, bladder cancer, nasopharyngeal carcinoma and colon cancer, and produced anti-proliferative effects on various tumor cells, not much research has been carried out on its inhibitory effects on breast cancer cell proliferation, and the mechanism involved [5-7].

The purpose of the present investigation was to determine the apoptotic and anti-proliferative influences of ginsenoside on MCF-7 cells, as well as the mechanism involved.

EXPERIMENTAL

Materials

Human papillary carcinoma MCF-7 cells were purchased from Nanjing Kaiji Biotechnology Development Co. Ltd. Ginsenoside (purity > 98.0%) was obtained from Chengdu Pulis Biotechnology Co. Ltd). Fetal bovine serum and DMEM were products of Hyclone Biological Company, USA, while MTT detection kits were purchased from AM-Resco, USA.

Apoptosis detection kits were bought from Shenzhen Jingmei Biological Engineering Co. Ltd. Phosphoglyceraldehyde dehydrogenase (GAPDH), p-PI3K, p-AKT and polyclonal antibodies were supplied by Santa Cruz, USA), while HRP-labeled 2° antibody was product of Beijing Zhongshan Jinqiao Biotechnology Co. Ltd.

Carbon dioxide (CO₂) incubator was bought from HeraCell 150i, flow cytometer was obtained from BD Company), while 2-16R cryogenic high-speed centrifuge was product of Hunan Henuo Instrument Equipment Co. Ltd. Inverted microscope was bought from Olympus. Protein electrophoresis and transfer equipment, Mini Opticon fluorescence quantitative PCR, and Enzyme marker were products of Bio-Rad, (USA).

Culturing of MCF-7 cells

Cell resuscitation

The MCF-7 cells in cryopreserved tubes at -80 °C were thawed by placing them in a beaker at 37 °C. The thawed liquid in each storage tube was then transferred into a 15-ml centrifuge tube, using a pipette gun, and the tube was centrifuged at 800rpm for 5 min. After centrifugation, 1ml of DMEM medium was put into the centrifuge tube and mixed with a pipette gun. The liquid from the centrifuge tube was aspirated into a cell bottle, and 3 mL of DMEM was added, followed by horizontal shaking. The surface of the cell bottle was wiped with alcohol-soaked cotton ball, and the cap of the cell bottle was loosened, followed by incubation of the cells in a 5 % CO₂ incubator at 37 °C. After 6 h, the cells were adherent to the wall, and the culture medium was refreshed.

Cell exchange

The old culture medium was replaced with 3 ml of PBS, followed by vigorous horizontal shaking and removal of medium. This operation was repeated twice. Then, an appropriate amount of DMEM complete medium was added, and the cell bottle surface was wiped with alcohol-moistened cotton ball, followed by loosening of the bottle cap and incubation of the cells at 37 °C in a 5 % CO₂ incubator.

Cell passage

When the cell growth density was about 80 - 90 %, the old culture medium was discarded, and 3 mL of PBS was added, followed by horizontal shaking and discarding of medium. This was repeated thrice. Then, 10 - 14 mL of 0.25 % trypsin was added to the remaining PBS in the stem cell bottle. The cell bottle was capped, and the bottle was shaken horizontally to ensure that the trypsin was evenly distributed at the bottom of the cell bottle.

The cell morphology was observed under the microscope, and digestion was stopped when the cells became round and bright. Then, trypsin was decanted and the lid was covered. Care was taken to ensure that the cells in the cell bottle were completely suspended in the medium. Then, 10⁻³ L of DMEM was put into the cell bottle. After mixing, 1 mL of the suspension was re-cultured in a fresh culture vial. An appropriate amount of DMEM was put into the two bottles, followed by incubation at 37 °C in a 5 % CO₂ incubator. Cell growth was observed 24 h later.

Determination of the effect of ginsenoside on cell proliferation

At 80 - 90 % confluence, the MCF-7 cells in good growth condition were subjected to digestion. Thereafter, they were plated in a 96-well plate at a concentration of 100 cells/well, and cultured for 24 h to make the cells completely adherent. After carefully pouring out the culture medium, 0.1 mL of new medium was added, prior to exposure to 0.02 mL of ginsenoside at different final concentrations of 30, 60, 90 $\mu\text{mol/L}$ (cells without ginsenoside served as control).

The wells were cultured in CO_2 incubator for 20, 40, and 60 h. At the end of each time period, 20 μL MTT solution was added to each well, and the culturing was continued for 4 h. Thereafter, the culture medium was carefully replaced with DMSO (150 μL) to solubilize the formazan crystals. The optical density of each well was read at a wavelength of 490 nm, and the percentage inhibition of cell proliferation was calculated.

Determination of apoptotic effect of ginsenoside

The MCF-7 cells at 80 - 90 % confluence were digested. A volume of 2 mL of each well was inoculated into a 6-well plate and cultured for 24 h to make it completely adherent to the wall. After carefully discarding the culture medium, 1.8 mL of new culture medium was added, and 200 μL of ginsenoside of different concentrations (final concentrations = 30, 60 and 90 $\mu\text{mol/L}$) were added, followed by culturing in CO_2 incubator for 48 h.

Cells without ginsenoside served as control. The cells were collected after tryptic digestion, and the concentration was changed to 5×10^5 cells/mL. Then, 1 mL of cell suspension was rinsed two times using PBS buffer. Then, 200 μL of cells was re-suspended in buffer. In the dark, 10 μL Annexin-V was added, and after 10 min, 5 μL of pyridine iodide was added. After 5 min, percentage apoptosis was measured using a flow cytometer.

Assay of protein expressions of p-pi3k and p-akt

After exposing the cells to different doses of ginsenoside for 48 h, they were rinsed thrice with PBS, and lysed with appropriate cell lysis solution. Total protein content of the lysates was determined with BCA method. Then, 20 μg of each sample was denatured and subjected to 12 % polyacrylamide gel electrophoresis. The protein bands were transferred to polyvinylidene membrane, and the membrane was incubated at 4 $^\circ\text{C}$ for 12 h with primary antibodies for p-PI3K, p-AKT and GAPDH, each at 1:500 dilution. Thereafter, the membrane was incubated with horse radish peroxide-linked 2^o antibody for 2 h. Following electrochemical luminescence for color development and exposure, optical densities of the protein bands were read in gel-pro analyzer.

Determination of ROS levels

Following treatment of MCF-7 cells with ginsenoside at different concentrations for 48 h, the cells were rinsed thrice with PBS, and incubated with 1 mL of 10 $\mu\text{L/L}$ DCFH-DA at 37 $^\circ\text{C}$ for 30 min. Then, the cover slides were clamped with plastic forceps, placed on the slide, and placed on normal fluorescence microscope.

Statistical analysis

Statistical analysis was done with SPSS 20.0 software. Measurement data are presented as mean \pm SD. Two-group comparisons were carried out using *t*-test. Counting data are presented as %, and were analyzed using χ^2 . Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

Effect of different doses of ginsenoside on viability of MCF-7 cells

As shown in Table 1, in control MCF-7 cells, there were no changes in viability. However, the % proliferation inhibition of cells treated with ginsenoside was markedly higher, relative to untreated cells, and it increased with extension of time and increase in concentration.

Table 1: Effect of ginsenoside at different concentrations on proliferation of MCF-7 cells (mean \pm SD)

Group	Concentration ($\mu\text{mol/L}$)	Proliferation (%)		
		20 h	40 h	60 h
Control	0	0	0	0
Low ginsenoside	30	13.26 \pm 1.05 ^a	28.41 \pm 3.12 ^a	35.18 \pm 3.20 ^a
Medium ginsenoside	60	26.95 \pm 3.26 ^{ab}	42.56 \pm 4.28 ^{ab}	56.84 \pm 5.08 ^{ab}
High ginsenoside	90	41.06 \pm 5.23 ^{abc}	58.46 \pm 6.38 ^{abc}	66.51 \pm 6.37 ^{abc}

^a $P < 0.05$, vs untreated; ^b $p < 0.05$, vs low ginsenoside cells; ^c $p < 0.05$, vs medium ginsenoside cells

Effect of different concentrations of ginsenoside on apoptosis of MCF-7 cells

Table 2 shows that % apoptosis in ginsenoside-exposed cells was markedly higher, relative to control cells, and it increased with increase in ginsenoside concentration.

Table 2: Effect of ginsenoside at different concentrations on apoptosis of MCF-7 cells (mean \pm SD)

Group	Concentration ($\mu\text{mol/L}$)	Apoptosis (%)
Blank	0	3.21 \pm 0.21
Control	30	21.31 \pm 2.16 ^a
Low ginsenoside	60	38.79 \pm 3.18 ^{ab}
Medium ginsenoside	90	48.15 \pm 5.41 ^{abc}

^a $P < 0.05$, vs with control; ^b $p < 0.05$ B, vs low ginsenoside group; ^c $p < 0.05$, vs medium ginsenoside group

Effect of ginsenoside at different concentrations on p-pi3k and p-akt levels

Table 3 and Figure 1 show that there were marked downregulations of these proteins in cells treated with ginsenoside relative to untreated cells, they decreased with increase in ginsenoside concentration.

Table 3: Influence of ginsenoside at different concentrations on p-PI3K and p-AKT levels in MCF-7 cells (mean \pm SD)

Group	Concentration ($\mu\text{mol/l}$)	p-PI3K	p-Akt
Blank	0	0.84 \pm 0.12	0.46 \pm 0.06
Control	30	0.66 \pm 0.08 ^a	0.33 \pm 0.04 ^a
Low ginsenoside	60	0.50 \pm 0.10 ^{ab}	0.27 \pm 0.03 ^{ab}
Medium ginsenoside	90	0.28 \pm 0.04 ^{abc}	0.15 \pm 0.01 ^{abc}

^a $P < 0.05$, vs untreated; ^b $p < 0.05$ B, vs low ginsenoside cells; ^c $p < 0.05$, vs medium ginsenoside cells.

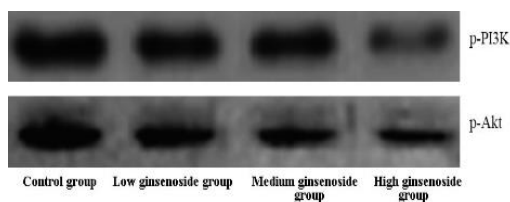


Figure 1: Effect of ginsenoside at different concentrations on p-PI3K and p-AKT in MCF-7 cells

Effect of ginsenoside at different concentrations on ROS expressions in MCF-7 cells

There were markedly lower concentrations of ROS in ginsenoside-treated MCF-7 cells than in control group, and their levels decreased with increase in ginsenoside concentration ($p < 0.05$), as shown in Figure 2.

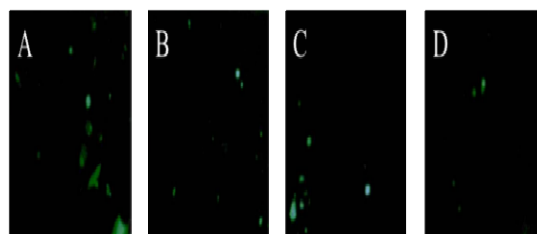


Figure 2: Influence of ginsenoside at different concentrations on ROS levels in MCF-7 cells. A: ROS expressions in MCF-7 cells in the control group; B: ROS in low ginsenoside group; C: ROS expressions in MCF-7 cells in the ginsenoside group, and D: ROS in high ginsenoside group

DISCUSSION

Studies have shown that variations in endogenous genetic susceptibility to breast carcinogenesis account for 20-25 % of breast cancer incidence, while more than 70% of breast cancer is caused by long-term exposure to environmental factors, accounting for 7 - 10 % of systemic tumors. The incidence of breast cancer is high among women aged 40 - 60 years (before and after menopause), and the incidence is on the rise [8,9]. At present, due to continuous progress in medical research, the treatment of breast cancer has improved, with chemotherapy as the most important treatment strategy after surgery and radiotherapy.

However, these treatments are often associated with undesirable side effects such as hair loss, vomiting, eating difficulties, pain, bone marrow suppression and drug resistance [10]. Therefore, it is necessary to evolve better treatment methods which can be used to achieve anti-tumor effects while improving the prognosis of patients [11]. Ginsenoside is the main active component of ginseng. Studies have shown that it inhibits tumor angiogenesis through inhibition of the proliferation and migration of vascular endothelial cells, inhibition of VEGF activity and its signal transduction pathway, and inhibition of the degradation of extracellular matrix [12].

The present study used different concentrations of ginsenoside to treat MCF-7 cells, and found

that the % inhibition of MCF-7 cell proliferation in the control group did not change with time. However, the % inhibition of cell viability was markedly higher in ginsenoside-exposed cells than in untreated cells, and it increased with time and ginsenoside concentration. The percentage of apoptosis in ginsenoside-treated MCF-7 cells was significantly higher than that in control group, and increased with increase in ginsenoside concentration. These results suggest that ginsenoside suppresses the viability of MCF-7 cells, and induces cell death in them.

It has been reported that ROS/PI3K/Akt signal route is vital for the regulation of cell viability [13]. The present study found that the enhancement of this signal route is among the causes of resistance of mammary cancer to hormone therapy. Studies have revealed that p-Akt, a serine/threonine protein kinase, p-PI3K (composed of regulatory p85 and catalytic subunits), and ROS (oxygen-containing chemically reactive molecules), promote breast cancer cell proliferation, increase cancer cell survival and tumorigenesis, and also promote angiogenesis, thereby enhancing metastasis of breast cancer cells.

Some researchers have reported that qinglong-coated polysaccharides directly inhibited or killed human colon cancer HCT-116 cells *in vitro*, through antitumor mechanisms related to ROS/PI3K/Akt signaling pathways [14-17]. In the present study, there were downregulations in p-Akt and p-PI3K in ginsenoside-exposed MCF-7 cells, relative to control cells, and their levels decreased with increase in ginsenoside concentration.

The expressions of ROS in ginsenoside-exposed cells were markedly decreased, relative to control cells, and decreased with increase in ginsenoside concentration, suggesting that the inhibitory effects of ginsenoside on proliferation of MCF-7 cells, and its apoptotic effects might be associated with regulation of the ROS/PI3K/Akt signaling route.

CONCLUSION

These results show that ginsenoside decreases the viability of MCF-7 cells and induces their cell death via suppression of ROS/PI3K/Akt signaling pathway. Due to certain limitations, including the small number of samples used in this study, it was not possible to determine a clear relationship between ginsenoside and ROS/PI3K/Akt signaling route. This will be addressed in subsequent studies.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Authors' contributions

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Zhongsheng Tong; Lihong He, Xiaorui Wang, Qing Ma, Weipeng Zhao, Yongsheng Jia, Guolei Dong, Yuehong Zhu, Xiaochen Jia and Zhongsheng Tong collected and analyzed the data while Lihong He wrote the manuscript. All authors read and approved the manuscript for publication.

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