

Antitumor activity of gossypol polyphenol is mediated through apoptosis induction and sub-G1 cell cycle arrest in SEG-1 human esophageal cancer cell line.

Ping Chen^{1,2}, Li Zhang³, Jing-Wen Li¹, Lei Tan⁴, Hong-Wei Xu^{1*}

¹Department of Gastroenterology, Provincial Hospital Affiliated to Shandong University, Jinan 250021, PR. China

²Department of Gastroenterology, Taian Central Hospital, Taian 271000, PR. China

³Department of Respiratory Intensive Care Unit, Taian Central Hospital, Taian 271000, PR. China

⁴Department of Thoracic Surgery, Taian Central Hospital, Taian 271000, PR. China

Abstract

The main objective of the present study was to evaluate the antitumor effects of gossypol in human esophageal cancer cells (SEG-1) by studying its effects on apoptosis induction and cell cycle phase distribution. Gossypol is a naturally occurring polyphenolic aldehyde with numerous biological activities. MTT assay was used to demonstrate the cytotoxic effect of gossypol while as fluorescence and scanning electron microscopic techniques were employed to study the effect of gossypol on cellular morphology. Flow cytometry was used to investigate the effects on cell cycle phase distribution and apoptosis induced by gossypol. Results showed that gossypol exhibited significant and time dependent cytotoxicity against the cancer cells. With increase in gossypol dose there was an increase in number of cells that were stained orange and red which is an indication of apoptosis. Gossypol treatment at lower doses have shown surface projections and blebbing of the membrane while as, at higher doses apoptotic bodies were found. In addition it has shown to induce sub-G1 cell cycle arrest.

Keywords: Esophageal cancer, Apoptosis, Gossypol, Anticancer activity.

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Introduction

Esophageal cancer is the sixth leading cause of cancer-related deaths and is the eighth most frequent cancer throughout the world. Around 450000 new cases and 400000 deaths from esophageal cancer are reported every year globally. In China, esophageal cancer accounts for about half of the total esophageal cancer incidences worldwide [1]. Esophageal cancer which affects more males than females has been recognized to have two main subtypes depending on the pathological characteristics. One type which is more dominant is the esophageal squamous cell carcinoma and the other which is less common is the adenocarcinoma. Because of the prospective features of invasion and metastasis in esophageal carcinoma cells, which are important prognostic factors, the overall 5-year survival rate is poor despite advanced treatment [2,3]. Despite many advances in treatment including radiotherapy, chemotherapy and surgery, the 5-year survival rate is low and the important reason for this treatment failure is the tumor recurrence and multidrug resistance [4,5]. Esophageal cancer usually remains undiagnosed till late stages, and advanced esophageal cancer is accompanied with poor outcome and is one of the most intractable of cancers. Thus,

there is a strong demand for new curative approaches to advanced esophageal cancer [6]. The aim of the present study was to investigate the antitumor activity of gossypol in human esophageal cancer cell line SEG-1. Gossypol, which is a naturally occurring polyphenolic aldehyde, is isolated mostly from the genus *Gossypium* plants. It has shown diverse biological activities. The present study demonstrates the effects of gossypol on apoptosis induction, cell cycle arrest, cell cycle-related proteins and mitochondrial membrane potential loss.

Materials and Methods

Materials

Gossypol (98% purity), Penicillin, Streptomycin, Rhodamine-123, Triton X-100 and 5% paraformaldehyde were purchased from Jinchan Biochemistry Company Ltd. (Anhui, China). All the reagents used in the experiments were of analytical grade. Fetal Bovine Serum (FBS), 2.5% trypsin, Dulbecco's modified Eagle's medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO were purchased from Gibco (Carlsbad, CA, United States). Annexin V-FITC and Acridine Orange (AO)

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was purchased from (Beyotime Institute of Biotechnology, Shanghai, China). Cell cycle phase determination kit was procured from Keygen Biotechnology (Nanjing, China).

Cell lines and cell culture conditions

SEG-1 human esophageal cancer cell line was procured from the Cell Institute, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin in humidified atmosphere with 5% CO₂ at 37°C. Further the cells were sub cultured after attaining 95-100% confluency.

MTT cell proliferation assay

The cytotoxicity of gossypol on human esophageal cancer cells was evaluated by MTT cell viability assay which is a colorimetric assay for assessing cell metabolic activity. In brief, SEG-1 cells were seeded in 96-well plates (1×10^5 cells/well). The cells were treated with various doses (0, 5, 10, 25, 75, 100 and 125 µM) of gossypol molecule for 12, 24 and 48 hours. After incubation, MTT dye (10 mg/ml) was added to each well and incubated for another 4 hours, further the formazan precipitate formed was dissolved in 400 µL DMSO, and then the absorbance was measured in Automated Microplated Reader (Bio-Tek, VT, USA) at 570 nm. The percentage of viable cells was calculated by the following formula:

$$\text{Inhibitory ratio (\%)} = (\text{OD control} - \text{OD treated}) / \text{OD control} \times 100\%.$$

Cytotoxicity was expressed as the concentration of gossypol inhibiting cell growth by 50% (IC₅₀ value).

Apoptotic assessment using fluorescence microscopy and acridine orange staining

Cells were seeded at a density of 2×10^5 cell/ml in a volume of 4 ml/well on sterile cover slips placed in 6-well tissue culture plates. After incubation, the medium was removed and replaced with fresh medium with 5% FBS. The cells were treated with gossypol (0, 10, 25 and 100 µM). Following drug treatment, the cover slips with monolayer of cells were inverted on the glass slide with 10 µL of AO stain (20 µg/ml). Images were captured using a UV fluorescence microscope (Olympus Optical Co., LTD, Tokyo, Japan) using UV filter at 400 X magnification to detect morphological changes due to apoptosis.

Scanning Electron Microscopy (SEM) studies of ultrastructural cellular morphology

SEG-1 cells at a density of 2×10^5 cells were seeded in six-well microtitre plates and were treated with (0, 10, 25 and 100 µM) concentrations of gossypol for 12 h. The cells were then centrifuged at 12,000 rpm for 20 minutes followed by Phosphate Buffer Saline (PBS) washing. The supernatant was removed and resuspended in 20 ml of 0.15 M cacodylate buffer. The sample was again centrifuged at 12,000 rpm for 10

min. The cacodylate buffer was removed and 1.2% of Osmium Tetroxide (OsO₄) in 0.2 M cacodylate buffer was added. The cover slips were washed three times with 0.2 M cacodylate buffer. After that 30% ethanol was added for 5 minutes. The coverslips were glued onto stubs with silver print and dried for about 20 minutes at room temperature. Then the samples were covered with gold using sputter coater. The images were captured by a scanning Electron Microscope (JOEL 64000, Japan) at accelerating voltage of 15-25 KV).

Annexin V-FITC binding assay/quantification of apoptotic cell death

In order to quantify and confirm whether gossypol induced apoptosis, Annexin V-FITC binding assay using flow cytometry was performed. Briefly, SEG-1 cells were treated with gossypol (0, 10, 25 and 100 µM) for 48 h, and then harvested by trypsinization. Harvested cells were then incubated in annexin V-FITC (25 µg/ml) and propidium iodide (25 µg/ml), at room temperature for 20 min in the dark, and analysed using BD FACSCalibur™ flow cytometer (BD Bioscience) taking a minimum of 15000 cells per sample.

Cell cycle analysis

Cell cycle analysis was performed by using FACSCalibur™ instrument (BD Biosciences, San Jose, CA). ModFit LT cell cycle analysis software was used to determine the percentage of cells in the various phases of the cell cycle. Briefly, SEG-1 cells (2×10^5) cells/well were treated with various doses of gossypol (0, 10, 25 and 100 µM) for 48 hours. Subsequently, the cells were collected, washed with ice cold PBS buffer, fixed with 70% alcohol at 4°C for 12 hours, and stained with propidium iodide in presence of 1% RNAase A at 37°C for 30 minutes prior to flow cytometric analysis.

Statistical analysis

All experiments were done in triplicate and the results are conveyed as the mean ± standard deviation. Graphpad Prism (Graphpad Software, Inc., CA, USA) was used for performing statistical analyses and P<0.05 was taken to designate a statistically significant difference.

Results

Antitumor activity of gossypol against SEG-1 cells

The chemical structure of gossypolis shown in Figure 1. MTT assay was used to assess the antitumor activity of gossypol in SEG-1 cells. Results revealed that gossypol exhibited a potent, concentration dependent as well as time-dependent growth inhibitory effect on the proliferation of SEG-1 cancer cells with IC₅₀ values 69.5, 49.2 and 31.3 µM at 12, 24 and 48 h time intervals respectively (Figure 2). IC₅₀ value gives an indication about the potency of the compound, higher its value lower will be the potency of the given drug.

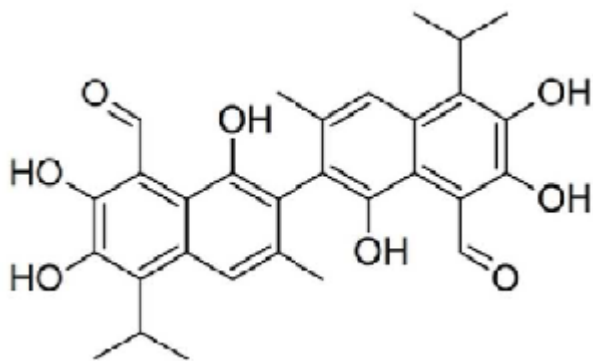


Figure 1. Chemical structure of gossypol.

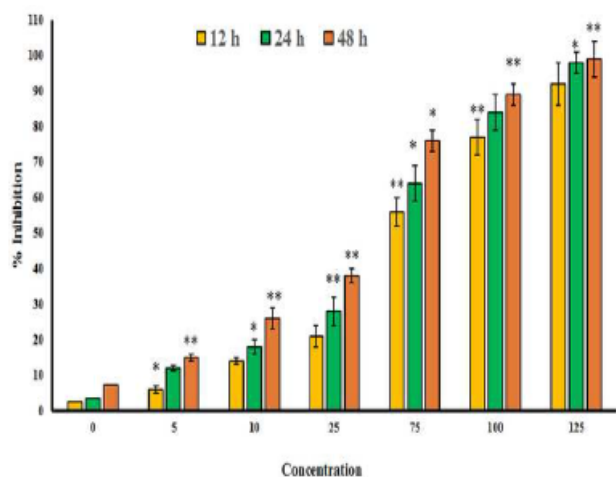


Figure 2. cytotoxic effect of gossypol in human esophageal cancer cells (SEG-1). Data are shown as the mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, vs 0 μ M (control).

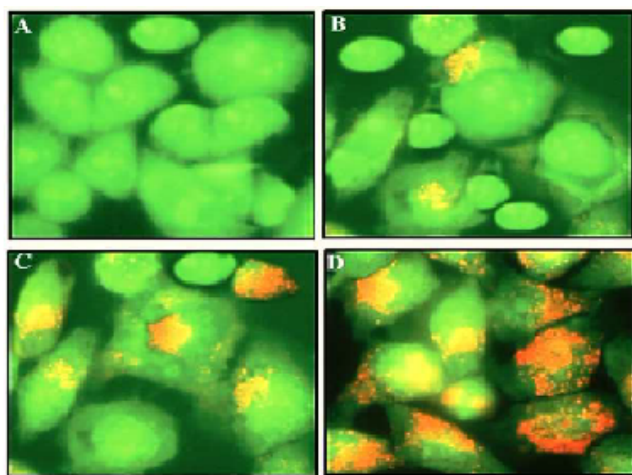


Figure 3. AO staining of SEG-1 cells using fluorescence microscopy. After treating cells with different doses of gossypol for 48 h, cells were stained with AO and observed under fluorescence microscope (400 X magnification). As compared to untreated control group (A), gossypol-treated cells (B-D) showed clear signs of early and late apoptosis. B, C and D represent effect of 10, 25 and 100 μ M dose of gossypol.

Apoptotic detection of SEG-1 cancer cells by fluorescence microscopy using AO staining

The results from this assay are shown in Figure 3 and indicate that viable cells with undamaged DNA show green fluorescence indicating normal cellular morphology while as apoptotic cells have damaged or fragmented DNA and stain as orange and red. Gossypol treatment in SEG-1 cells showed reduction in the number of viable cells significantly and in a dose-dependent manner. With an increase in the dose of Gossypol, an increasing number of cells were stained orange and red indicating apoptosis process happening within the cells. Gossypol-treated cells displayed characteristic features of apoptosis including membrane blebbing, chromatin condensation and apoptotic body formation which were not detected in untreated cells (Figure 3A).

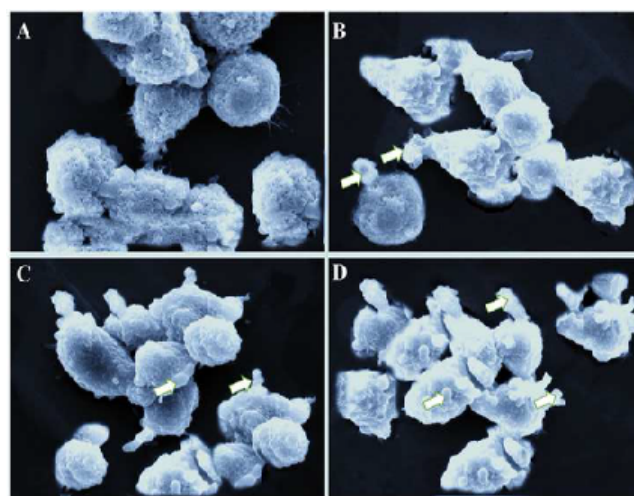


Figure 4. Scanning electron microscopy in SEG-1 cells. A represents untreated cells; B, represents cells treated with gossypol at 10 μ M for 48 h; C, represents cells treated with 50 μ M and D; represents cells treated with 100 μ M. The untreated control cells exhibited rough surface and microvilli. The treatment for 48 h caused reduction in cell size, blebbing of the plasma membrane and treatment at 100 μ M for 48 h, transformed the cells into apoptotic bodies (magnification 2000 X).

SEG-1 esophageal cancer cell surface analysis by SEM

Scanning electron microscopy is used to reveal the morphological features of the cell surface. The apoptosis process in a cell usually leads to cell surface changes including shrinkage, membrane blebbing, loss of microvilli structures and smoothing. As can be seen from Figure 4A, untreated SEG-1 cells were rounded with normal surface. However, gossypol treatment at lower dose led to various surface projections and blebbing of the membrane. At higher gossypol concentration, apoptotic body formation was witnessed (Figure 4 B-D). Overall, scanning electron microscopy study clearly showed that gossypol resulted in the formation of apoptotic body formation in human esophageal cancer cells.

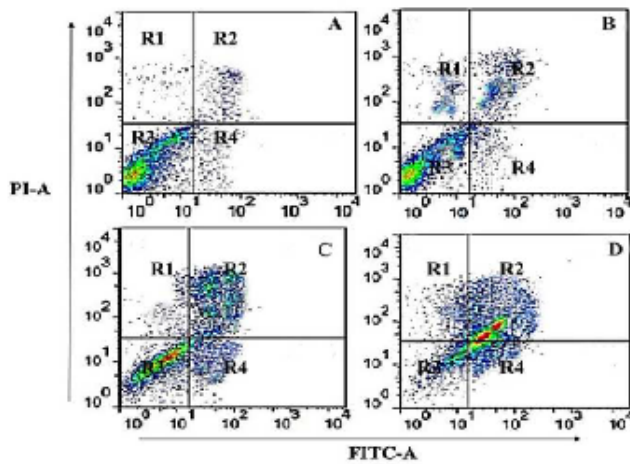


Figure 5. Annexin V-FITC assay-based quantification of gossypol induced apoptosis in human esophageal cancer cells. The cells were treated with gossypol (0, 10, 50 and 100 μM) for 48 h and analyzed by flow cytometry. The different quadrants R1, R2, R3 and R4 represent necrotic cells, late apoptotic cells, viable cells and early apoptotic cell population respectively. Percentage of apoptotic cells increases from 3.3% in control cells (A), to 24.5%, 39.9% and 56.3% in 10 μM (B), 50 μM (C) and 100 μM (D) gossypol-treated cells respectively.

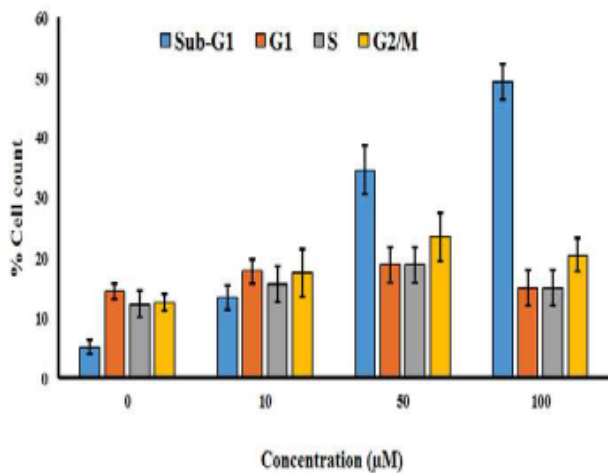


Figure 6. Gossypol induced sub-G1 cell cycle arrest in SEG-1 cells. The cells were treated with 0, 10, 50 and 100 μM dose of gossypol for 48 h, stained with propidium iodide and then analyzed by flow cytometer (FACS Calibur flow cytometer). ModFit LT cell cycle analysis software was used to determine the percentage of cells in the various phases of the cell cycle.

Gossypol-induced Apoptosis quantification using annexin V-FITC assay

In order to quantify the extent of apoptosis induced by gossypol in human SEG-1 cells, annexin V-FITC assay was carried out using flow cytometry. The results revealed that gossypol induced both early and late apoptosis in a concentration-dependent manner. As compared to the untreated control cells (Figure 5A), gossypol treated cells at 10, 50 and 100 μM led to potent apoptosis induction (Figure 5 B-D). The four quadrants R1, R2, R3 and R4 represent necrotic cells, late

apoptotic cells, viable cells and early apoptotic cell populations respectively. Percentage of apoptotic cells increases from 3.3% in control cells (A), to 24.5%, 39.9% and 56.3% in 10 μM (B), 50 μM (C) and 100 μM (D) respectively.

Gossypol induced sub-G1 cell cycle arrest in SEG-1 cells

The effect of gossypol on cell cycle phase distribution was evaluated by using flow cytometry using propidium iodide staining. The cells were treated with various doses of gossypol (0, 10, 50 and 100 μM) for 48 h and then subjected to flow cytometric detection. The results indicated that gossypol induced potent and dose-dependent increase in the percentage of cells in sub-G1 phase of the cell cycle. This was also accompanied by a small increase in the percentage of G2/M cells also (Figure 6).

Discussion

The results of the current study demonstrates that gossypol exerts potent and dose-dependent anti-proliferative effects in SEG-1 cells. The cytotoxic effects induced by gossypol were found to be time-dependent, as the antiproliferative effect was shown to increase with increase in time intervals after gossypol treatment. Acridine orange staining of the SEG-1 cells followed by fluorescence microscopic examination revealed that gossypol treatment led to DNA fragmentation and the cells were stained orange and red. Gossypol-treated cells displayed characteristic features of apoptosis including membrane blebbing, chromatin condensation and apoptotic body formation which was not detected in untreated cells. Scanning electron microscopy revealed that gossypol treatment induced alterations in cell surface morphology. On treating cells with increasing doses of gossypol, several cellular surface projections and loss of microvilli structures and surface smoothing was observed. Apoptotic body formation in gossypol-treated cells can be easily seen in SEM analysis. Further, annexin V-FITC assay quantified the percentage of apoptotic cells after gossypol treatment indicating the increase in percentage of apoptotic cells from 3.3% in control cells, to 24.5%, 39.9% and 56.3% in 10 μM (B), 50 μM (C) and 100 μM (D) respectively. Gossypol also induced potent and dose-dependent increase in the percentage of cells in sub-G1 phase of the cell cycle which was further accompanied by a small increase in the percentage of G2/M cells also.

Gossypol has been reported to have several biological properties including antimalarial and proapoptotic properties due to the regulation of Bax and Bcl-2 genes [7]. It has also been reported to inhibit replication of HIV-1 virus [8]. Some authors report that gossypol is an effective protein kinase C inhibitor. This compound has also been reported to induce apoptosis in ovarian cancer cells through the mediation of oxidative stress. It has also shown to induce apoptosis in human prostate cancer cells via the downregulation of Bcl-2 and upregulation of Bax at the mRNA and protein levels. Gossypol also activated caspases-3, 8 and 9 and increased PARP [poly (ADP-ribose) polymerase] cleavage. Gossypol has

been identified as a BH3-mimetic inhibitor of pro-apoptotic Bcl-2 family members, including Bcl-2, Bcl-xL, and Mcl-1, and induces apoptosis in various types of cancer [9-13]. To the best of our knowledge, anticancer activity of gossypol against human esophageal cancer (SEG-1) cells has not been reported till date. As such, the present report constitutes the first such report on this type of human cancer.

Conclusion

The findings of the present study reveal that gossypol inhibited cancer cell growth in human esophageal tumor cell lines via the induction of apoptosis and sub-G1 cell cycle arrest.

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*Correspondence to:

Hong-Wei Xu

Department of Gastroenterology

Provincial Hospital Affiliated to Shandong University

Jinan 250021

PR. China

Email: xuhongweihw@hotmail.com