The Antitumoral and Biochemical Effects of Gossypol on Human Cervix Cancer Cell Line ME-180

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ABSTRACT

Gossypol, a polyphenolic compound extracted from cotton plants, has been found to be an anticancerogenic agent. The aim of our study was to investigate whether gossypol induced cell death on ME-180 cervix cancer cells, and whether it was a potent inhibitor of some antioxidant enzymes, like catalase, glutathione reductase and glutathione-S-transferase.

The cells were incubated with four different doses (5,10,15 and 20 μM) for 24, 48 and 72 hours. After gossypol treatment, the cytotoxic effects were measured with MTT tests. Using DNA agarose gel electrophoresis, cellular internucleosomal DNA fragmentation of the cells treated with gossypol and untreated were examined. Consequently, gossypol caused different fragmentation on tumour cells due to apoptosis. Gossypol was found to be a potent inhibitor of catalase, glutathione reductase and glutathione-S-transferase.

Key Words: Gossypol, Apoptosis, Catalase, Glutathione reductase, Glutathione-s-transferase

ÖZET

Gossypol'ün İnsan Serviks Kanser Hücre Hattı ME-180 Üzerindeki Antitümoral ve Biyokimyasal Etkileri

Pamuk bitkisinden izole edilen polifenolik bir bileşik olan gossypol'ün antikarsinojenik bir ajan olduğu bulunmuştur.Çalışmamızın amacı Gossypol'ün ME-180 serviks hücrelerinde hücre ölümünü indükleyip indüklemediğini ve katalaz, glutatyon redüktaz ve glutatyon-S-transferaz gibi antioksidan enzimler için potansiyel bir inhibitör olup olmadığını araştırmaktır

Hücrelere 4 farklı dozda (5,10,15 ve 20 μM) ve 24,48 ,72 saat süre ile gossypol uygulaması yapılmıştır. Gossypol uygulamasından sonra sitotoksik etkiler MTT testi ile değerlendirilmiştir. DNA agaroz jel elektroforez tekniği kullanılarak gossypol uygulanan ve uygulanmayan hücrelerdeki internükleozomal DNA fragmentasyonu tespit edilmiştir. Sonuç olarak Gossypol, apoptozis yüzünden tümör hücreleri üzerinde farklı fragmentasyonların oluşmasına yol açmıştır. Gossypol'ün katalaz, glutatyon redüktaz ve glutatyon-S-transferaz için potansiyel bir inhibitör olduğu bulunmuştur.

Anahtar Kelimeler: Gossypol, Apoptozis, Katalaz, Glutatyon redüktaz, Glutatyon-s-transferaz

INTRODUCTION

Gossypol, [(1, 1', 6, 6', 7, 7'- hexahydroxy 5-5'-diisopropyl – 3, 3'-dimethyl (2, 2'-binaphthalene) 8, 8'-dicarboxaldehyde)] which contains many hydroxyl and methyl groups, is a yellow, polyphenolic aldehyde and is extracted from cotton seeds (1) (Figure 1).

It was originally identified as a male antifertility agent and has been used as an effective male contraceptive drug for many years . Tuszynski and Cossu found its anticarcinogenic effects against several tumor cell lines grown in tissue culture (2). Recently, gossypol has been shown to inhibit the growth of several tumour cell lines such as; Ehrlich ascites tumor cells (3), P388 and L1210 murine leukemias (4), SW-13 adrenocortical carcinoma cells (5), murine erythroleukemia cells (clone 6A11A) (6), human glioma cell line HS683, U373, U87 and U138 (7), hormone-dependent and hormone-independent breast carcinoma cells MCF-7 (drug sensitive), MCF-7 Adr (multidrug-resistant), MDA-MB-231 and T47D (8-11), melanoma cell line SK-mel-19 and SK-mel-28 (12), promyelocytic leukemia cell line HL-60 (13,14), colon carcinoma cell line HT-29 and Lo Vo (15), breast carcinoma cell line T47D and ovarian carcinoma cell line OV-CAR-3 (16,17) in vitro and it is found to be potent against proliferating melanoma cell lines and its activity was studied in pigmented and non-pigmented cell lines (18).

There is a report indicating that gossypol is unlikely to be useful in patients with advanced cancer (19). All these studies agreed that gossypol is tolerated and clinically safe (2).

Gossypol is an agent which inhibits oxidative phosphorilation. It has been found that gossypol disturbs energy metabolism in dividing tumour cells and therefore these cells could not required energy for themselves. In gossypol-treated cells, gossypol tends to disturb mitochondrial related metabolic process rather than interfering with DNA synthesis (20). Treatment with gossypol inhibits DNA synthesis of the cells (9), causes DNA breaks or DNA fragmentation (21). And also mechanism of gossypol induced anti-tumour activity, appears to be cell type specific. However, how gossypol induces tumour cell death is still not clear.

Gossypol inhibits a wide variety of cytosolic and mitochondrial enzymes important for cell growth, including protein kinase C (22,23), calmodulin, (22), lactic dehydrogenase (24), topoisomerase II (25), DNA polymerase (26), malate dehydrogenase (27), glutathione-s-transferase (22, 27-29).

In the present study, we investigated the cytotoxic effects of gossypol on ME-180 cervix cancer cell lines and its effects on three kind of antioxidant enzymes such as catalase, glutathione reductase and glutathione-S-transferase. The other aim of our study was to investigate whether gossypol induced apoptosis in ME-180 cell line or not.

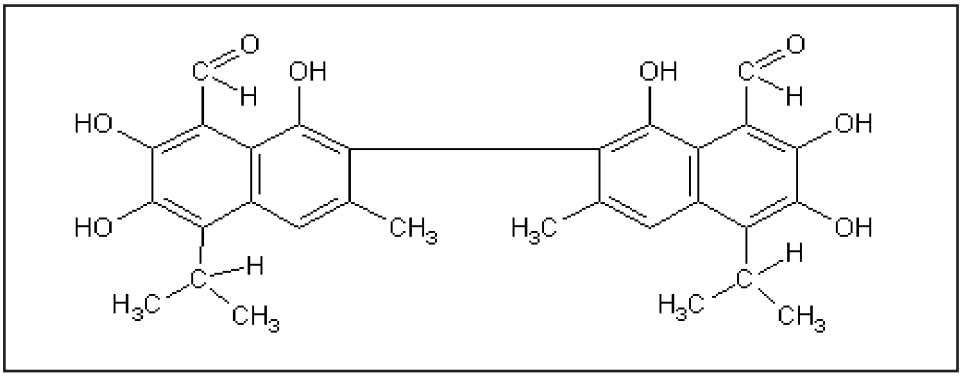

Figure 1. Chemical structure of gossypol

MATERIAL AND METHODS

Cell Culture and Gossypol Treatment

The human cervix cancer cell line ME-180 obtained from American Type Culture Collection were grown in Corning plastic flasks (25 cm2) in 5% CO_2 in air at 37^{0}C. The growth medium was Dulbecco's modified Eagle's medium (Sigma) supplemented by 10% heat-inactivated fetal calf serum (Sigma), 200mM L-glutamine (Sigma) and 1% antibiotic-antimycotic mixture (Sigma). Gossypol (Sigma) dissolved in absolute ethanol, was added to the culture during logarithmic growth phase at different concentrations 5, 10, 15 and 20 μM for 24, 48 and 72 hours. Absolute ethanol alone was added to the control flasks, so that the ethanol concentration in the experimental and control flasks was 0.1%.

Determination of Cell Viability

The cytotoxic effect of gossypol on human cervix cancer cells ME-180 was determined using the MTT (Sigma) (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) viability assay. Cells (3×10^4) were seeded in 96-well flat-bottomed plates and treated with different gossypol concentrations (5, 10, 15 and 20 μM). The cells were incubated in 37^{0}C, 5 % CO_2 humidified incubator for 24, 48 and 72 hours. At the end of incubation period, the medium was replaced with 20 μl of 5mg/ml MTT in phosphate- buffered saline. The cells were incubated for two hours and then MTT was removed. 100 μl DMSO (Dimetyl sulfoxide) (Sigma) was added on the cells and the cells were reincubated for two hours . At the end of this incubation, the optic density of the samples were measured at 540 nanometer wave lenghts by spectrofotometry. Cell viability was calculated using the following formula;

Cell Viability (%) = 100-A_1/A_0

Where A_1 and A_0 are the absorbances obtained from treated and untreated cells, respectively. The mean value of tree experiments was used for analysis (30).

Agarose Gel Electrophoresis

DNA fragmentation was analysed by agarose (Sigma) gel electrophoresis as described previously (31). Fragmented DNA from cell extracts was loaded onto 2% agarose gels and visualised by ethidium bromide.

Protein Assay

The amount of total protein was determined by the Lowry et al. procedure, using bovine serum albumine as a standard (32).

Enzyme Assays

Catalase activity was measured using the method of Luck (33). Glutathione reductase activity was measured using the method of Carlberg and Manervik (34). Glutathione-S-transferase activity was measured by using the method of Habig and Jacoby (35).

Statistical Analysis

By using SPSS 11.0 (Statistical Package for Social Sciences, SPSS Inc., Chicago, USA) software, all the data obtained from triple repeated experiments for enzyme studies and cell viability were statistiscally evaluated with non-parametric Kruskal-Wallis and Mann-Whitney U tests.

RESULTS

Cytotoxic Effects of GP

The in vitro sensitivity of ME-180 cervix cancer cell line to gossypol was determined by using the MTT, a viability assay. In all experimented doses of gossypol, death rate has increased related to time. As shown in Figure 2, at 24, 48 and 72 hours respectivly, the death rate has been observed as 28 %, 66%, 92 % for 5 μM; 24%, 79 %, 89 % for 10 μM; 12%, 71%, 81 % for 15 μM; 3 %, 70 %, 79% for 20 μM ($p<0,001$) (Figure 2).

Agarose Gel Electrophoresis

DNA fragmentation was assessed by agarose gel electrophoresis as a qualitative method. It has been detected that related to time gossypol has also increased DNA fragmentation of ME-180 cells paral-

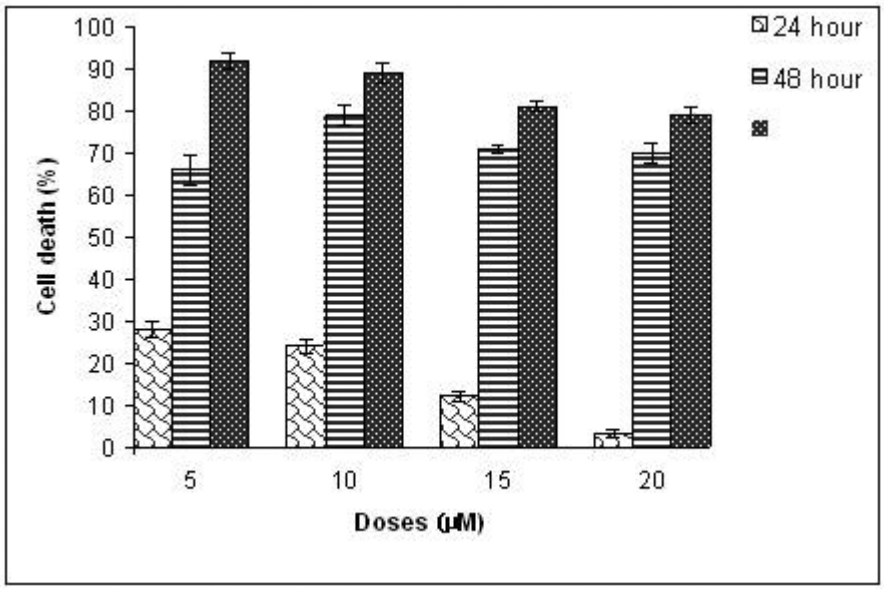

Figure 2. The percentage of cell death at different doses for 24, 48 and 72 hours. Results are represantative of three independent experiments ($p < 0.001$).

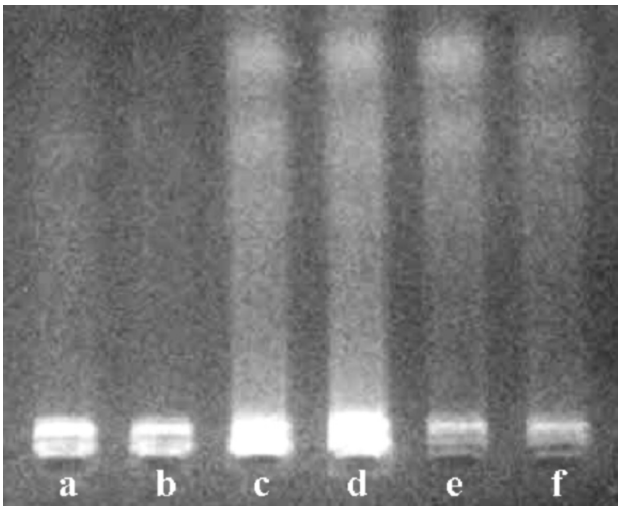

Figure 3. Genomic DNA fragmentation of ME-180 cells at the end of 72 hours (a) ethanol control, (b) untreated cells, (c) 5 μM, (d) 10 μM, (e) 15 μM, (f) 20 μM.

lel to its cytotoxic effects on ME-180 cells. Ethanol control and untreated cell cultures did not exhibit any fragmented DNA, as expected (Figure 3).

Enzyme Assays

At the end of 24 hours, when compared to control cells, the catalase activity has incresed in 5, 10, 15 μM dose of gossypol treated ME-180 cells; however the activity has decreased in 20 μM dose of gossypol ($p < 0.001$). Due to dose increase, respectful amount of decrease has been observed both in glutathione reductase and glutathione-S-transferase activities ($p < 0.001$) (Table1).

At the end of 48 hours, gossypol has caused substantial loss in the catalase activity in 5, 10, 15 μM doses and there was no activity in 20 μM dose whereas, related to dose increase reduction has been caused both in glutathione reductase and glutathione-S-transferase activities ($p < 0.001$) (Table 2).

At the end of 72 hours, gossypol has induced substantial decrease in the activities of all three enzymes ($p < 0.001$) (Table 3).

DISCUSSION

Since 1970, though studies have concentrated on the contraceptive effects of gossypol, recently more interest has been emphasized on its antitumoral effects. It has been known that gossypol has antip-

Table 1. The enzymes activities at different doses of gossypol at the end of 24 hours.

Tests Groups (İM)	Catalase (μmol/mgtot.pro)	Glutathione Reductase (μmol/mgtot.pro)	Glutathione-stransferase (μmol/mgtot.pro)
Control	11.45 ± 0.2553	0.131 ± 0.0012	0.311 ± 0.0041
5 μM	30.01 ± 3.312	0.0710 ± 0.00748	0.206 ± 0.1517
10 μM	22.81 ± 1.764	0.023 ± 0.0041	0.1680 ± 0.0102
15 μM	14.920 ± 3.363	0.014 ± 0.0014	0.0660 ± 0.0061
20 μM	6.87 ± 0.9583	0.0065 ± 0.00316	0.0325 ± 0.1138

Table 2. The enzymes activities at different doses of gossypol at the end of 48 hours.

Tests Groups (İM)	Catalase (μmol/mgtot.pro)	Glutathione Reductase (μmol/mgtot.pro)	Glutathione-stransferase (μmol/mgtot.pro)
Control	61.07 ± 0.502	0.301 ± 0.0032	0.361 ± 0.0013
5 μM	13.20 ± 0.1706	0.160 ± 0.0184	0.1129 ± 0.0161
10 μM	3.148 ± 0.0757	0.123 ± 0.0325	0.072 ± 0.0151
15 μM	1.89 ± 0.0635	0.063 ± 0.0269	0.019 ± 0.0106
20 μM	0	0.038 ± 0.0088	0.012 ± 0.00048

roliferative and antimetastatic effects on different cancer cells (3-17). Gossypol was shown to be the most potent inhibitor of keratinocyte proliferation in the anti proliferative MTT assay, and a potent anti oxidant (9,36). Gossypol-induces cell cycle arrest on G0/G1 phase as well as cell apoptosis of HT-29 cells (2). Gossypol has induced apoptosis by inhibiting protein kinase C in doses of 50, 100 μM for human premyelocytic leukemia HL60 cell line (13).

Gossypol generally exhibits many biological activities by disturbing cellular energy metabolism which are glycolisis, mytocondrial oxidative phosphorilation and electron transport (7). It has been known that gossypol can inhibit some enzymes as glutathione-S-transferase, DNA polymerase, protein kinase C, calmoduline stimulated C-AMP phosphodiesterase and adenylate kinase which are not dehydrogenase and reductase (11). There has been no prior study about the effects of gossypol on antioxidant enzymes like catalase and glutathione reductase actitivites.

In this study, gossypol has been assessed in four different doses (5, 10, 15 and 20 μM) on ME-180 cells and its effect on DNA fragmentation, and also enzyme activities has been observed for 24, 48 and 72 hours.

The cytotoxic effects of gossypol and fragmentation has exhibited parallelisation. The most cytotoxic effect of gossypol on the cells has been detected at the end of 72 hours. In accordance with this result, the most fragmentation has been realised at the end of 72 hours. The fragments of the DNA indicate that gossypol induces apoptosis.

The results of this study are in general agreement with these findings with other studies which have shown that gossypol has an anti-apoptotic activity.

It has been observed that gossypol has increased catalase activity in the cell line only at the end of 24 hours whereas, reduced the same activity at the end of 48 and 72 hours. The increase of catalase activity by gossypol at the end of 24 hours can be considered as if gossypol can produce radicals in cancer cells. There has not been observed any kind of increase in glutathione reductase and glutathione-S-transferase activities in any amount of doses ($p < 0.001$). There has not been any report about the ef-

Table 3. The enzymes activities at different doses of gossypol at the end of 72 hours.

Tests Groups (İM)	Catalase (μmol/mgtot.pro)	Glutathione Reductase (μmol/mgtot.pro)	Glutathione-stransferase (μmol/mgtot.pro)
Control	141.50 ± 0.756	0.249 ± 0.0040	0.590 ± 0.0037
5 μM	23.40 ± 1.238	0.0187 ± 0.00212	0.251 ± 0.00304
10 μM	11.43 ± 0.812	0.0179 ± 0.002.4	0.127 ± 0.00635
15 μM	3.352 ± 1.026	0.0090 ± 0.00054	0.050 ± 0.0158
20 μM	0.249 ± 0.0040	0.0014 ± 0.00014	0.024 ± 0.0087

fect of gossypol on catalase and glutathion reductase. Our results have shown that gossypol inhibits the activity of these enzymes in dose and time dependent manner. Hence, our study complies with other studies which have shown that gossypol inhibits glutathione-S-transferase.acvtivity (24, 26).

Since the ME-180 model representative of human cervix cancer, the results of these and future experiments could provide valuable information for clinicans in the treatmant of cancer.

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