

Research Paper:

A Comparative Study of the Cytotoxic Effects and Oxidative Stress of Gossypol on Bovine Kidney and HeLa Cell Lines



Mahsa Daneshmand¹, Jamileh Salar Amoli^{1*}, Tahereh Ali Esfahani²

1. Department of Comparative Bioscience, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

2. Department of Basic Sciences, Toxicology and Animal Poisoning Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.



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ABSTRACT

Background: Cotton seed is one of the main sources of protein in animal feeds, containing gossypol, which has been shown to have toxic effects. Results reported by various studies also indicate the anti-cancer effects of gossypol on various cell types. However, its toxic effects on human and animal cells have not been fully established. This study was planned to investigate, for the first time, the cytotoxic effects and oxidative stress induced by gossypol on normal Bovine Kidney (BK) and HeLa cell lines, representing typical healthy and cancer cells, respectively.

Methods: The BK and HeLa cell lines were treated for 24, 48 or 72 hours with 5, 10 or 20 ppm of gossypol (+/-). The cellular bio-availability and cytotoxicity were measured by MTT assay. The catalase and Malondialdehyde (MDA) levels were also measured to represent the oxidative stress parameters.

Results: The percentages of cytotoxicity in BK and HeLa cell lines were calculated at a gossypol concentration of 5, 10 and 20 ppm over 24, 48 or 72 hours of incubation, respectively. The Lethal Concentration 50 (IC₅₀) values were also determined for the two cell lines. No changes in the catalase and lipid peroxidase activities were observed in either cell line.

Conclusion: The percentage of the gossypol cytotoxicity was concentration-dependent. By comparing the IC₅₀ in both cell lines using one-way Analysis of Variance (ANOVA) analysis, a significant difference was observed, suggesting that HeLa cells were less sensitive to gossypol than the BK cells. Lack of changes in the oxidative stress, as tested by catalase and MDA assays, demonstrated that gossypol did not induce oxidative stress in either cell line.

Keywords: Bovine Kidney (BK) cells, Catalase, Malondialdehyde (MDA), Cervical Cancer, Gossypol, *In Vitro*

* Corresponding author:

Jamileh Salar Amoli, PhD.

Address: Department of Comparative Bioscience, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

E-mail: salaramolijamile@gmail.com

Introduction

Gossypol is a terpenoid [1] or a yellow-color polyphenol aldehyde phytotoxin that is synthesized in the roots of Malvaceae plants, *Thespesia populnea*, and especially in the seeds of cotton genus, *Gossypium herbaceum* and *Gossypium hirsutum*. Its environmental distribution and concentration are de-

pendent on the number of pigment glands [2]. Gossypol was first isolated by Longmore in 1886 from *Gossypium* plants, hence the origin of its name, "*Gossypol*", coined by Marchlewski in 1899 [3]. Gossypol has two enantiomers (+/-). Based on various toxicity studies, the (-) enantiomer is more cytotoxic and potent [4] than the (+) counterpart. In biochemistry terms, gossypol has different derivatives and tautomer's, each with its specific chemical and toxicity properties [5]. The chemical struc-

ture of gossypol is impressive due to its toxic side chains. In this study, we used the gossypol (+, -) types. It has two aldehyde and six hydroxyl functional groups, causing rapid reaction with ether and ester products [5], and amino acids in proteins. In 2011, Hong li [6] reported that gossypol reduced the expression of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) in Human breast cancer cell line. In 2012, a study on retinoblastoma cells demonstrated that gossypol inhibited the activities of these tumor cells [7]. In another study on ovarian cancer cell line, the oxidative stress by gossypol induced cell apoptosis [8].

Gossypol exposure to humans and livestock occurs orally. It is distributed in the body in two forms: a) as free gossypol and b) as bound to proteins [9]. The free form of gossypol is more toxic and accumulates in cardiac muscle, causing toxic necrosis [10, 11]. The protein-bound gossypol is often found in the liver and kidneys [10]. Also, gossypol has its disadvantages. For example, it reduces iron ions [12] and essential amino acids (Lysin) absorption and inhibits pepsin and trypsin enzymes in the gastrointestinal tract of animals, leading to protein indigestion [13, 14]. It has been known that non-ruminant animals are more sensitive to gossypol than the ruminants. There is no report of acute gossypol toxicity in humans and there are a few studies on normal cell lines; although the long-term consumption of cotton seeds oil by young Chinese couples has reportedly led to infertility [15].

Gossypol affects enzymatic and mitochondrial functions, leading to cellular apoptosis. It reduces lactate dehydrogenase isoenzymes and malathion dehydrogenase but inhibits glutathione transferase [16]. In the pancreatic cancer cells, the gossypol (-) type inhibits cytochrome c, Bcl-2 homologous antagonist/killer (Bak) and Bax activities, causing damages to cristae and fragmentation of mitochondria [17]. Gossypol is antagonist to BH3 (Bcl-2 homology (BH) domains) by binding to specific proteins and inducing cell apoptosis [18]. In cells resistance to apoptosis, it causes cell death secondary to oxidative stress [17]. In such situations, antioxidants, i.e., catalase and glutathione peroxidase inhibit its oxidative process. The properties of gossypol have led scientists to investigate its antitumor properties. In this study, we explored the oxidative stress and cytotoxic effects of both gossypol (+, -) on HeLa cells, a cancer cell line, and normal BK cells. We also compared the data in an effort to enrich the existing information about the gossypol cytotoxicity and oxidative stress properties.

Materials and Methods

Human cervical cancer cell line (HeLa) and Bovine Kidney cell line (BK) were obtained from the Iranian Razi institute. Gossypol (+, -), Dimethyl Sulfoxide (DMSO), and thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma Aldrich (St. Louis, MO, USA). Penicillin/streptomycin, trypsin/EDTA, Fetal Bovine Serum (FBS) and Dulbecco's modified eagle medium (DMEM) were purchased from Gibco Laboratories (Gaithersburg, MD, USA).

Cell culture: Both BK and HeLa cell lines were cultured to confluence in DMEM media in 60ml tissue culture dishes at 37°C in a humidified incubator. The culture media contained 4 mM L-glutamine, supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (v/v) and 1% penicillin/streptomycin in salt solution (100 U/ml and 0.1 mg/ml, respectively) [19].

In vitro toxicity assay: cells were cultured in 96-well plates at a density of 5x10⁴ cells/well, and incubated at 37°C overnight. They were then treated with varying concentrations of (+, -) gossypol and incubated for 24, 48 or 72 hours. For MTT test, the cell culture media removed with a pipette, 100µL thiazolyl blue tetrazolium bromide was added to the wells and incubated for another three hours. Then, 100 µL of Dimethyl Sulfoxide (DMSO) was added to each well and incubated for 15 minutes. Finally, the absorbance of the solution in the wells were read at 570 nm with an ELISA reader (Awareness, USA) [20]. The following formula was used for calculating the percentage of cell toxicity: 1-mean of absorbance (at each concentration)/mean absorbance of blank x 100 [21]. The Lethal Concentration 50 (IC₅₀) values were calculated, using the following formula: (0.5-b)/a [22].

Catalase assay: The catalase assay (EC 1.11.1.6) was performed according to the method published by Chaoui, et al. in 1997 [23]. The disappearance of H₂O₂ was evaluated by measuring the decline in the absorbance at 240 nm of the reaction mixture, containing 100µL H₂O₂. The molar extinction coefficient was 36.6 M⁻¹ x cm⁻¹. The reaction mixture containing cells plus 100 µL H₂O₂ was brought up to a final volume of one mL by adding potassium phosphate buffer at pH 7.0.

Lipid peroxidation: The lipid peroxidation was evaluated, using thiobarbituric colorimetric assay. For this purpose, one mL thiobarbituric solution (67%) was added to 100 µL of the cells, and the mixture was incubated at 95°C for 30 minutes. The reaction was stopped by placing the tubes on ice. Then one mL n-Butanol (Merck,

USA) was added to the tubes, shaken and were centrifuged at 3500g for 10 minutes. The absorbance was read at 532nm on a spectrophotometer (Beckman, USA) [24].

Statistical analysis: The data were analyzed using Excel-10. All data points were derived from the average of at least five independent trials and expressed as the mean \pm standard error of the mean. The statistical significance of the differences between each control and the relevant treatment group was determined, using one-way Analysis of Variance (ANOVA) and the correlation between the sets of two parameters tested.

Results

The data representing the cytotoxicity of gossypol at varying incubation periods and at the three concentrations for BK and HeLa cells are shown in Table 1. The increasing cell toxicity of (+/-) gossypol against BK and HeLa cell lines correlated consistently with rising the (+/-) gossypol concentration. With longer incubation times, the gossypol toxicity increased steadily. It should be noted that with HeLa cell line at all concentrations of (+/-) gossypol, the highest cell toxicity happened with 48-hr incubation.

IC₅₀ values: Table 2 shows the IC₅₀ values for both cell lines. The values increased slightly with an increase in incubation time; however, it declined suddenly between 24 and 48 hours of incubation. The IC₅₀ values grew again slowly at 72 hours of incubation for BK cell line. Figure 1 illustrates the IC₅₀ variations for both cell lines. The IC₅₀ values for HeLa cells at all incubation times were greater than those for the BK cells, indicating their higher resistance to gossypol than the BK cell line.

Lipid peroxidation assay: For this purpose, a linear calibration graph was drawn and the levels of malondialdehyde versus standards were determined, using one-way analysis of variance ($P < 0.05$). There was no significance difference between the two cell lines for the lipid peroxidation property.

Catalase assay: Gossypol (+/-) did not induce catalase activity in neither cell line, but there were some insignificant variations in the catalase activity for HeLa cells at 10ppm with 24-hr incubation based on the one-way analysis of variance.

Discussion

In this study, we verified the cell toxicity and oxidative stress activities for gossypol (+/-) on BK and HeLa cell lines, and compared the variables between the two cell groups. In both cell lines, with a rise in the gossypol concentration, the cell toxicity increased, and there was a correlation between the gossypol (+/-) concentration and the degree of cytotoxicity. Our results were consistent with those reported previously by other studies on different cell lines [25, 26]. It is noteworthy that most of the cell toxicity in BK cells occurred with the gossypol at 10ppm and over 48 hours of incubation. Similarly, the highest cell toxicity for HeLa cells occurred at 48 hours of incubation. Therefore, it was evident that the cell lines investigated in this study were sensitive to gossypol (+/-) at 10ppm or higher.

HeLa cells have been used widely in numerous in vitro studies for years [27], and they are resistant to apoptosis, chemicals and shocks. In this study, the IC₅₀ value was the lowest at 24 hours of incubation. However, longer incubation times in previous studies have shown lower

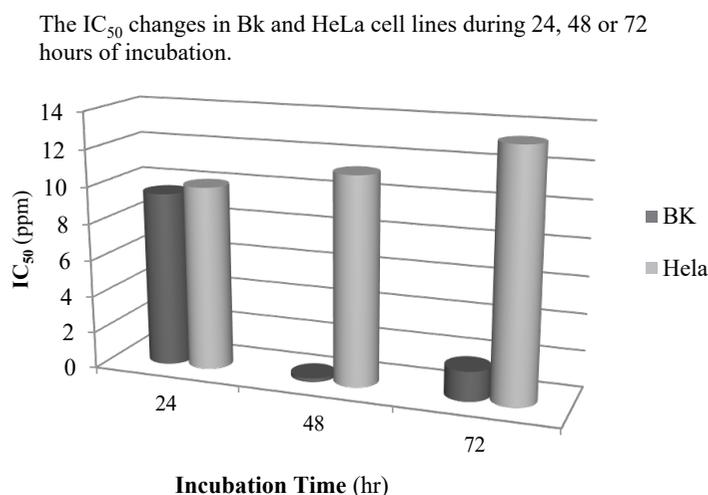


Figure 1. The IC₅₀ in BK and HeLa cell lines versus incubation time

Table 1. The cell toxicity percentages of gossypol in BK and HeLa cell lines

Cell Line	Incubation Time (hours)	Gossypol Concentration (ppm)	Cell Toxicity (%)	Correlation
Bovine Kidney (BK)	24	5	39	0.9
		10	43	
		20	92	
	48	5	53	0.9
		10	88	
		20	95	
	72	5	60	0.9
		10	69	
		20	97	
HeLa	24	5	3	0.9
		10	46	
		20	94	
	48	5	26	0.9
		10	39	
		20	92	
	72	5	16	0.9
		10	20	
		20	88	

IC₅₀ values. Based on the statistical correlation test, the IC₅₀ value in HeLa cells correlated with the incubation period. In BK cells, the lowest IC₅₀ values were observed at 48 hours of incubation. In this cell line, the IC₅₀ for 72 hours of incubation was greater than that of the 48 hours but the IC₅₀ values for 72-hr incubation were less than those for the 24 hours. In this respect, there was a

significant difference between the two cell lines, Upon ANOVA analysis,

Figure 1 illustrates the IC₅₀ values for both BK and HeLa cell lines, and compares them based on the cellular characteristics. It is evident that the IC₅₀ in BK cells is less than that for HeLa cells, suggesting that the BK

Table 2. The IC₅₀ of gossypol in BK and HeLa cell lines

Cell Line	Incubation Time (hr)	IC ₅₀	Correlation
Bovine Kidney (BK)	24	9.51	0.8
	48	0.2	
	72	1.62	
HeLa	24	10.02	0.9
	48	11.26	
	72	13.32	

cells are more sensitive to the toxic effect of gossypol over 48-hour incubation. At this point, it is not known why cells became resistant to gossypol. Some cells, especially the HeLa cells, become resistant to antitumor drugs, the strongest reason being the high expression of membrane proteins which can also occur in normal cells [28]. The IC_{50} values may vary based on the cell line under study, the gossypol (+/-) concentration range and the experimental method. For example, the IC_{50} value for human leukemia cell line has been reported to be $4.5\mu M$ [29] while the values for a subline of the ubiquitous keratin-forming tumor cell line have been $5.7\mu M$ [30]. The oxidative stress parameters did not change significantly based on the ANOVA analysis ($P < 0.05$), there was no significant differences between MDA concentrations in each of the two cell lines compared with the standards. Previous studies conducted on oxidative stress due to gossypol (+/-) toxicity provided differing results in 2018. That study reported that gossypol (+/-) caused oxidative stress, resulting in reduced testosterone release [31]. In another study, the gossypol's oxidative stress induced cell death in ovarian cells [8]. Earlier in 2015, researchers demonstrated that the gossypol (+/-) consumption caused reproductive system deficiency, likely due to its oxidative stress [32]. Finally, Hou, et al. has argued that gossypol (+/-) does not increase free radicals release and catalase cannot stop the apoptosis induced by gossypol [33].

Conclusions

The results of this study provided evidence that gossypol (+/-) did not induce oxidative stress in BK and HeLa cell lines. Further, variations in the IC_{50} values varied depending on the cell lines. Finally, the gossypol toxicity against the cells used in this study was concentration-dependent.

Our findings have confirmed the effects of gossypol (+/-) on normal cells compared to a human cancer cell line. Based on our findings, it can be concluded that gossypol (+/-) at 10ppm induced the highest toxic effect on these cells.

Limitation of the study: Because of the current political situation in Iran, we could not obtain some of the needed biochemical materials, including different kits for analyzing oxidative stress markers and apoptosis on four cell lines. This limited the interpretation of the oxidative stress results.

Recommendation for future studies: Further studies are recommended to explore the effects of gossypol on

other oxidative stress markers and the adverse effects on mitochondria in various cell lines, and the associated mechanisms of cell death.

Ethical Considerations

Compliance with ethical guidelines

All of the institutional guidelines on research ethics were observed in this study as set by the Animal Poisoning Research Center of the Faculty of Veterinary Medicine at Tehran University, and the study's protocol was granted approval by the Ethics Committee (Ethics Code: 93; Protection Code: 7506008/6/13).

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Author's contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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