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Gossypol and endometrioma cells
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·Original Article·

Gossypol inhibits proliferation of endometrioma cells in culture

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Abstract

Aim: To evaluate the anti-proliferative activity and mitochondrial toxicity of gossypol in endometrioma cells maintained in short-term cultures. **Methods:** (A) Three endometrioma cell lines from patients were treated with 25 or 50 nmol/L gossypol for up to 12 days. The effect of gossypol on the cell growth was recorded. (B) A phosphorescence oxygen analyzer was used to determine the effects of gossypol on mitochondrial oxygen consumption of six endometrioma cell lines from patients. (C) Cellular gossypol accumulations in three endometrioma cell lines from patients were measured by high-pressure liquid chromatography. **Results:** Proliferation of the endometrioma cells was inhibited by 25 and 50 nmol/L gossypol. Respiration of the endometrioma cells was inhibited by 10 μmol/L gossypol. Cellular gossypol was detected in the endometrioma cell lines that were treated for 24 h with 10 and 0.3 μmol/L gossypol. **Conclusion:** Gossypol invokes a potent toxicity on cultured endometrioma cells. *(Asian J Androl 2007 May; 9: 388–393)*

Keywords: gossypol; endometrioma; mitochondria; oxygen consumption; cellular respiration

1 Introduction

Gossypol [(2,2'-binaphthalene)-8,8'-dicarboxyaldeyde-1,1'6,6',7,7'-hexahydoxy5,5'-di-isopropyl-3,3'-dimethyl] is a yellow pigment found in the cottonseeds of the plant genus Gossypium [1]. This polyphenolic binaphthalene aldehyde has potent contraceptive and anticancer activities, including those against multi-drug resistant tumors [2–6]. Its mechanism of action however remains under investigation.

Gossypol was introduced (in ~1960) as an anti-fertility agent for men [4, 7]. This effect was found to be dose dependent [8, 9]. Animal and human studies showed gossypol reduced sperm counts and increased dead-sperm numbers. These toxic insults were mostly attributed to a direct effect of gossypol on the seminiferous tubules of the testes [10]. The spermatids and spermatocytes were especially sensitive, showing increased drug accumulation and ultrastructural changes in the nuclear membranes, endoplasmic reticulum and mitochondria. *In vitro* studies on the mitochondria showed gossypol specifically inhibited respiration [11].

Gossypol was also used in China (since ~1970) for

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treatment of women with uterine fibroids, uterine bleeding and endometriosis [12]. The treatment in these cases was effective in reducing the size of fibroids, leading to amenorrhea and improving the symptoms of endometriosis.

Gossypol primarily impairs mitochondrial functions [6]. The drug uncouples oxidative phosphorylation [13, 14] and initiates apoptosis. The latter process involves release of cytochrome c from the mitochondrial intermembrane space and activation of caspases that inhibit cellular respiration [15, 16].

The present study evaluates the effects of gossypol on endometrioma cells in culture. The results show toxic effects of gossypol on endometrioma cells.

2 Materials and methods

2.1 Endometrioma cells

Six endometrioma cell lines (BP1295, KF681, VG5800, SH391, SH3134, and MCL10275) were developed from 6 patients. The tissues were collected during operative laparoscopy or laparotomy for endometriosis with associated endometrioma. Each patient signed a consent document for the study, which was approved by the Institutional Review Board for the protection of human subjects.

A 1–2 cm piece of each tissue was excised and immediately sent to the research laboratory. The remaining specimen was sent to surgical pathology for histologic studies. The latter tests confirmed the diagnosis of endometrioma in the 6 patients studied [17].

2.2 Endometrioma cell culture

Epithelial and stroma cells were isolated from the tissue using 0.1 mg collagenase (LS00414; Worthington Biomedical Corp., NJ, USA) per mL DPBS (Mediatech, Inc., Westwood, MA, USA). Tissues were digested in a 37ºC water-bath with periodic agitation for 15 min. The cells were then washed with media (M199 with Earle's salts and L-glutamine; Mediatech Inc., Westwood, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were centrifuged for 5 min at 800 × *g*. The cell pellet was re-suspended in 10 mL of the above media. The cells were counted and plated at $1–2 \times 10^6$ cells per mL on T-25 cm flasks (3038; Falcon Products, Morristown, TN, USA) that were coated with 10 µg/mL fibronectin. The cultures were incubated at 37ºC in an incubator containing 5% CO_2. The media were changed every three days. The cells were grown in culture until confluence, and then passed to another culture. The cells were frozen in the above media with 8% dimethylsulfoxide (Sigma-Aldrich Inc., St. Louis, MO, USA) at $2–3 \times 10^6$ cells/mL. Aliquots (1.5 mL each) of the cell suspensions were placed in 2-mL cryovials and stored in liquid nitrogen [17].

2.3 Cell proliferation

Thawed frozen stroma cell lines were plated at 3×10^4 viable cells per well on fibronectin-coated (FLPP, Sigma-Aldrich Inc., St. Louis, MO, USA) 6-well plates (3046; Falcon Products, Morristown, TN, USA). On day 3, the media (described above) were exchanged with 25 or 50 nmol/L gossypol along with a control (media without gossypol) that was run simultaneously. The cells were harvested and counted on days 3, 6, 9 and 12 using a hemocytometer. Viability was done using trypan blue exclusion stain.

Gossypol solution was freshly prepared in cold 95% ethanol and kept on ice. The concentration of gossypol was determined by ultraviolet absorbance at 385 nm, using a molar extinction coefficient of 18 000 [18].

2.4 Respiration

Cellular respiration was measured at 25ºC in sealed vials containing $\sim 1–5 \times 10^6$ cells per condition [18, 19]. The cells were suspended in 0.5 mL Pd phosphor solution (RPMI medium containing 10 mmol/L glucose, 2 µmol/L Pd phosphor [Porphyrin Products Inc., Logan, UT, USA) and 3% fat-free albumin [final pH, ~7.4]). The solution was freshly made and stirred at 25ºC for ~30 min prior to use. Mixing was with the aid of a ceramic stir bar. Rate of respiration was the negative of the slope of $[O_2]$ *vs. t* (zero-order rate, in µmol/L O_2 per min per 10^6 cells). The drift (Pd phosphor solution without cells) was 0.28 ± 0.05 µmol/L O_2 per min.

2.5 Instrument

$[O_2]$ in solution was determined as function of *t* using the Pd phosphor. The phosphorescence decay of the probe was characterized by a single exponential; the decay time (τ, disappearance of the phosphorescence signal) was inversely proportional to O_2 concentration [18, 19]. Samples were exposed to ten light flashes per sec from a pulsed light-emitting diode array with peak output at 625 nm (OTL630A-5-10-66-E, Opto Technology Inc., Wheeling, Il, USA). Emitted phosphorescent light was detected by a Hamamatsu photomultiplier tube

(#928; Stratford, CT, USA) after passing through a wide-band interference filter centered at 800 nm (Omega Optical Inc., Brattleboro, VT, USA). The amplified phosphorescence decay was digitized at 1 MHz by a 20 MHz A/D converter (Computer Board Inc., Middleboro, MA, USA). Two hundred fifty samples were collected from each decay curve and the data from 10 consecutive decay curves were averaged for calculating. [O_2] was calculated using: $\tau^0/\tau = 1 + \tau k_q [O_2]$; where τ, is the lifetime in the presence of oxygen; τ^0, the lifetime in the absence of oxygen; and k_q, the second-order O_2 quenching constant for the Pd phosphor in the presence of fat-free albumin (k_q, ~4.1×10^8 M^{-1} s^{-1}).

2.6 Cellular gossypol accumulation

Cells (in M199 media plus 10% FBS) were incubated with gossypol for 24 h at 37ºC in an incubator containing 5% CO_2. The cell pellets were then rinsed with drug-free media and collected by centrifugation (1 000 × *g* for 2 min). The cell pellets were suspended in 200 µL of acetonitrile/HCl/ascorbic acid solution. The mixtures were vigorously mixed and place in boiling water for 2 min. The supernatants were then separated on HPLC and gossypol peak was detected by absorbance at 254 nm [20].

The analysis was performed on Beckman HPLC system (Beckman Coulter Inc.,Fullerton, CA, USA). The solvent was acetonitrile : H_2O : acetic acid (7:2:1) [20]. The column (4.6 × 250 mm Beckman ultrasphere IP) was operated isocratically at 1 mL/min. Standards (10 µmol/L gossypol in the acetonitrile/HCl/ascorbic acid solution) were included with each analytical run. The standard curves were linear ($r > 0.99$) over 5–30 nmoles.

3 Results

3.1 Gossypol inhibited the growth of endometrioma cells

The proliferations of 3 endometrioma cell lines (BP1295, KF681 and VG5800) were studied. Gossypol inhibited the growth of all 3 tumor cells. However, the sensitivity of the cells to the drug varied. The 1st tumor showed mild growth inhibition at 25 nmol/L and complete inhibition at 50 nmol/L (Figure 1A). The 2nd and 3rd tumors were similar, showing milder inhibition at either 25 or 50 nmol/L (Figure 1B and C).

3.2 Gossypol impaired endometrioma cell respiration

The respirations of 6 endometrioma cell lines (BP1295, KF681, VG5800, SH391, SH134 and MCL10275) were studied. In 3 tumors (the same cell lines in Figure 1), the cells were treated with 50 or 300 nmol/L gossypol for 24 h. Respiration was then measured at the end of the treatment period. Representative runs for the cell line VG5800 are shown in Figure 2. Respiration was unaffected by this gossypol treatment (Table 1).

We next investigated the respiration of another 3 cell lines (SH391, SH134 and MCL10275) during continuous exposure to 10 µmol/L gossypol. Respiration was decreased by ~40–75% in the presence of 10 µmol/L gossypol (Table 2).

3.3 Cellular gossypol accumulation

Table 1. Gossypol effects on cellular respiration. Three endometrioma tumors were studied. The cells were plated at ~5.5×10^5 per condition on day 3. On day 0, the media were exchanged for experimental media containing indicated concentrations of gossypol and simultaneously run with control media. The cells were harvested at 24 h and suspended in 0.5 mL Pd phosphor solution (RPMI medium containing 10 mmol/L glucose, 2 µmmol/L Pd phosphor and 3% fat-free albumin). Cellular respiration was measured at 25ºC in sealed vials. The rates of respiration (µmmol/L O_2 per min per 106 cells) were the negative of the slopes of [O_2] *vs. t*.

Cell lines	Gossypol treatment	Rate of respiration (µmol/L O_2 $min^{-1}/10^6$ cells)
BP1295	None	3.6
	50 nmol/L for 24 h	5.5
KF681	None	3.9
	50 nmol/L for 24 h	3.7
VG5800	None	7.2
	300 nmol/L for 24 h	7.2

Table 2. Gossypol effects on cellular respiration. Three endometrioma tumors were studied. Cells (10^6 cells per run) were suspended in 0.5 mL Pd phosphor solution (RPMI medium containing 10 mmol/L glucose, 2 µmol/L Pd phosphor and 3% fat-free albumin) with or without 10 µmol/L gossypol. Cellular respiration was measured at 25ºC in sealed vials. The rates of respiration (µmol/L O_2 per min per 10^6 cells) were the negative of the slopes of [O_2] *vs. t*.

Cell lines	Rate of respiration (µmol/L O_2 $min^{-1}/10^6$ cells)	
	No addition	10 µmol/L gossypol
SH391	4.2	1.4
SH3134	3.8	2.2
MCL10275	3.8	1.0

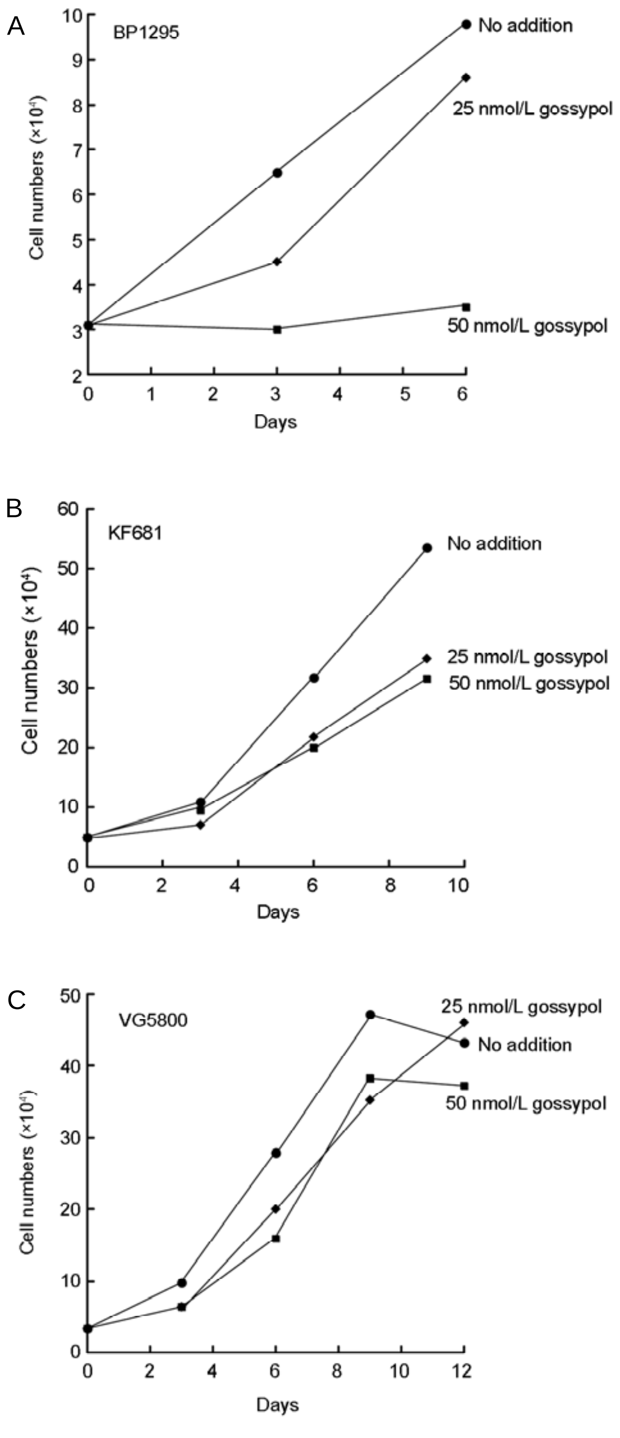

Figure 1. Gossypol inhibited proliferation of the endometrioma cells in culture. Three endometrioma tumors were studied. The cells were plated at ~5.5 × 10^5 per condition on day 3. On day 0, the media were exchanged for experimental media containing indicated concentrations of gossypol and simultaneously run with control media. The control and gossypol-containing media were changed daily. The cells were then harvested and counted as shown.

Table 3. Gossypol accumulation in endometrioma cells. Three endometrioma tumors were studied. The cells were plated at ~5.5 × 10^5 per condition on day 3. On day 0, the media were exchanged for experimental media containing indicated concentrations of gossypol and simultaneously run with control media. The cells were harvested at 24 h and analyzed for gossypol contents as described in methods.

Cell lines	Gossypol treatment	Cellular gossypol (μmol/L O_2 $min^{-1}/10^6$ cells)
BP1295	50 nmol/L for 24 h	N.D.
KF681	10 μmol/L for 24 h	25.0
VG5800	0.3 μmol/L for 24 h	13.9

Cellular gossypol was detected in the 2 endometrioma cell lines, KF681 and VG5800 that were treated for 24 h with 10 and 0.3 μmol/L gossypol, respectively (Table 3).

4 Discussion

The contraceptive effect of gossypol was discovered by the observation that the use of uncooked cotton seed oil was responsible for the high rate of infertility in various villages in China [21]. These reports appeared in the Chinese literature as early as 1933. Since then, studies have showed marked suppression of sperm production by gossypol.

Gossypol was also used for treatment of endometriosis, uterine fibroids, adrenal tumors and gliomas [5, 22]. The drug was successful (in ~90% of the cases) in reducing the sizes of endometrial cysts and fibroids [12]; but ~80% of the treated patients suffered from amenorrhea. There was no drug effect on gonadotropin or ovarian steroid hormones, suggesting the amenorrhea was related to a direct effect of gossypol on the endometrium [23]. Some investigators demonstrated reduction in estrogen and progesterone receptors in the endometrium of women treated with gossypol before they underwent hysterectomy [24].

The present study demonstrates an inhibitory effect of gossypol on the proliferation of three endometrioma cell lines in culture. The effect of gossypol on these endometrioma cells is thus similar to that on sperm [25].

The results also suggest that gossypol may have potent cytotoxic activity against endometriomas. The drug inhibits proliferation of endometriosis and interferes with cellular metabolism and mitochondrial functions. Further studies are necessary to address whether gossypol

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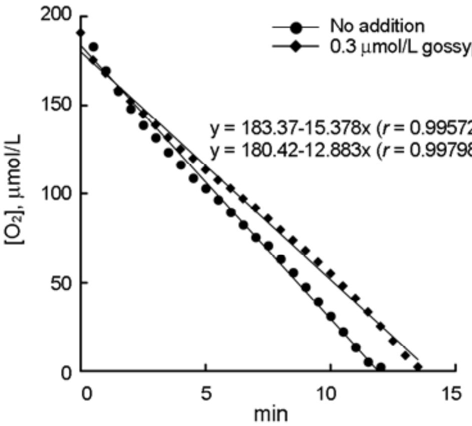

Figure 2. Representative experiment showing endometrioma cell respiration. The endometrioma cells (VG5800) at near confluence were incubated at 37ºC with gossypol for ~24 h. At the end of the incubation period, the cells were harvested and counted. Cellular mitochondrial oxygen consumption was then measured as described in Methods. Cell viability is 90% in cell line without gossypol and 82% in cell line with 0.3 μmol/L gossypol. Rates of respiration (in μmol/L O_2 per min) were determined as the negative of the slopes of the curves of $[O_2]$ *vs. t* (r > 0.99, Table 1).

is applicable to the treatment of endometriosis and fibroids.

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