

Gossypol induces DNA strand breaks in human fibroblasts and sister chromatid exchanges in human lymphocytes in vitro

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SUMMARY The male contraceptive agent gossypol was found to induce a dose related increase of DNA strand breaks in human fibroblasts in vitro at concentrations of 5 to 40 $\mu\text{g/ml}$. The effect was reduced in the presence of 2% fetal calf serum. A weak but reproducible increase in the SCE frequency was found in human lymphocytes treated for 1 hour in serum-free medium with 0.04 to 4 $\mu\text{g/ml}$ of gossypol.

Gossypol, a phenolic compound isolated from the cotton plant, is used as a male contraceptive agent in China. It causes a reversible inhibition of spermatogenesis and the side effects are reported to be mild and infrequent.^{1,2} However, the mechanisms of action of gossypol are poorly understood³⁻⁷ and its genetic toxicity has not been thoroughly investigated.⁸⁻¹¹ In this work we have studied the effect of gossypol on the induction of DNA strand breaks in cultured human fibroblasts and sister chromatid exchange (SCE) in human lymphocytes in vitro.

Materials and methods

Gossypol-acetic acid, methyl methane sulfonate (MMS), and bromodeoxyuridine (BrdU) were obtained from Sigma Chemical Co; Eagle's MEM, medium 199, Hank's balanced salt solution, and fetal calf serum from Flow Laboratories; phytohaemagglutinin (PHA) from Wellcome; hydroxylapatite (Bio-gel HTP) from Bio-Rad Laboratories; and methyl-tritiated thymidine (5 Ci/mmol) from the Radiochemical Centre, Amersham. Other chemicals were of analytical grade and obtained from local commercial sources.

DNA STRAND BREAKS ANALYSIS IN HUMAN FIBROBLASTS

Cultured human skin fibroblasts (6th to 12th passage) obtained from healthy donors were grown on glass plates and DNA was labelled by addition of methyl-tritiated thymidine (2 $\mu\text{Ci/ml}$ medium), as described previously.¹²

The cells were incubated in 1.5 ml Dulbecco's phosphate buffered saline (PBS) with gossypol (5 to 40 $\mu\text{g/ml}$ added in 8 μl dimethylsulphoxide (DMSO)) at 37°C for 30 minutes. Incubations were terminated by washing the cells in ice cold PBS. In some incubations, the cells were exposed to gossypol for 30 minutes, washed with Hank's balanced salt solution, and the incubation was continued using complete Eagle's MEM supplemented with 10% fetal calf serum.

DNA strand breaks were determined by the method of Ahnström and Erixon¹³ modified as described previously.¹² The cells were incubated in the dark in 1.5 ml of 0.03 mol/l NaOH—0.97 mol/l NaCl on ice. This treatment will cause lysis of the cells and denaturation of the DNA followed by DNA unwinding starting at strand breaks. After 30 minutes the samples were neutralised to pH 6.8, sonicated, and sodium dodecyl sulphate (SDS) (2.5 mg/ml) was added. Single and double stranded DNA were separated by hydroxylapatite chromatography, and the radioactivity in each fraction was determined by scintillation counting. Alterations in the number of DNA strand breaks are expressed as the percentage of total radioactivity eluted in the single strand fraction. In this series of experiments 19.2% \pm 5.4 of the DNA from solvent treated controls eluted in the single strand fraction. This experimental background is related to the denaturation and incubation conditions used.^{14,15}

SISTER CHROMATID EXCHANGE ANALYSIS IN HUMAN LYMPHOCYTES

Freshly collected heparinised blood from healthy donors was used. PHA stimulation and culture

conditions were as described previously.¹⁶ After 20 hours at 37°C the cells were washed with culture medium 199 and incubated for 60 minutes at 37°C with different concentrations of gossypol (added in DMSO) in serum-free medium 199. Control cultures received DMSO only. After 60 minutes of incubation the cells were washed and the culture continued in medium 199 supplemented with 25% fetal calf serum and 100 µmol/l BrdU. The total culture time was 75 hours. Two hours before harvest 0.125 µg/ml of colchicine was added. The harvest, staining of cells, and analysis of SCE were as described previously.¹⁶ The rate of cell replication in the cultures was estimated by scoring 100 cells and counting the numbers of cells in first, second, and third division, which are easy to distinguish owing to their differential staining properties after growth in BrdU.¹⁶

Results

Chemically induced DNA damage may result in DNA strand breaks,¹⁸ which can be detected by alkali treatment of cells followed by hydroxyl apatite chromatography to separate single strand DNA and double strand DNA.¹² An increase in the amount of single strand DNA eluted indicates DNA strand scission.¹⁴ In human fibroblasts treated with 5 to 40 µg/ml of gossypol in PBS for 30 minutes, the fraction of single strand DNA showed a dose-related increase (fig 1), indicating that gossypol causes DNA strand breaks.

To determine the effect of serum on the genotoxic effects of gossypol, fibroblasts were incubated in Eagle's MEM with gossypol (20 µg/ml) for 30 minutes in the presence and absence of 2% fetal calf serum. As shown in table 1, the fraction of single strand DNA was reduced to the background level when serum was added. In similar experiments with PBS, bovine serum albumin (50 mg/ml) also reduced the fraction of single strand DNA to background values in gossypol exposed fibroblasts (data not shown). These experiments indicate that the genotoxic effect of gossypol is reduced by binding to serum proteins. No reduction of gossypol induced DNA strand breaks was found when superoxide dismutase (0.3 µg/ml), dithiothreitol (5 mmol/l), or α-tocopherol (100 µmol/l) were added to the incubations (data not shown).

To study the persistence of the induced DNA breaks, fibroblasts were incubated with gossypol (20 µg/ml) in PBS at 37°C. After 30 minutes the cells were washed and the incubation was continued in Eagle's MEM supplemented with 10% fetal calf serum. No reduction in the fraction of single strand DNA was observed during a 5-hour incubation (fig 2).

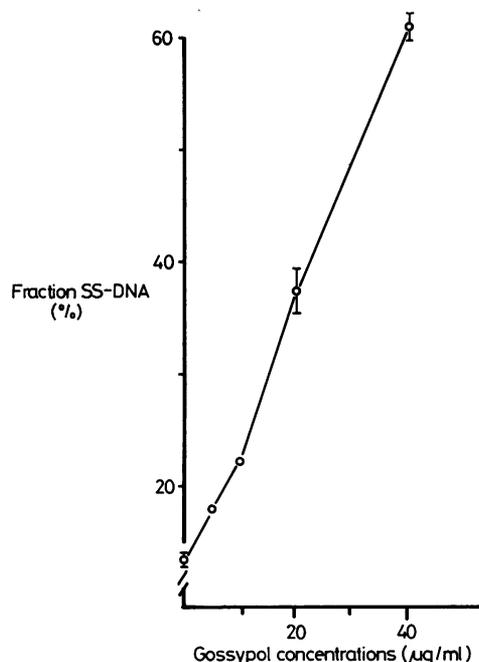


FIG 1 Induction of DNA strand breaks by gossypol. Human fibroblasts were incubated with different concentrations of gossypol for 30 minutes at 37°C. The fraction SS-DNA eluted and range of duplicate determinations are given.

The SCE inducing effect of gossypol was studied in human lymphocytes. In a preliminary experiment, gossypol was added to lymphocyte cultures in serum-containing medium. At a concentration of 40 µg/ml, gossypol caused severe growth inhibition and there was no significant effect on the SCE frequency at this or lower concentrations (not shown). This experiment confirms the results of Tsui *et al*¹¹ in showing a very small SCE inducing effect of gossypol treatment in serum-containing medium. In later experiments, gossypol treatment was carried out in serum-free medium. It was then found that the concentrations of gossypol used in the previous experiment were exceedingly toxic and that cell growth was completely inhibited. Further studies

TABLE 1 Effect of serum on gossypol induced DNA strand breaks in cultured human fibroblasts. Mean values and range of duplicate experiments are given.

	% SS-DNA eluted	
	No addition	+2% FCS
DMSO	24.0 ± 1.8	23.8 ± 3.3
Gossypol 20 µg/ml	55.8 ± 1.9	24.5 ± 0.1

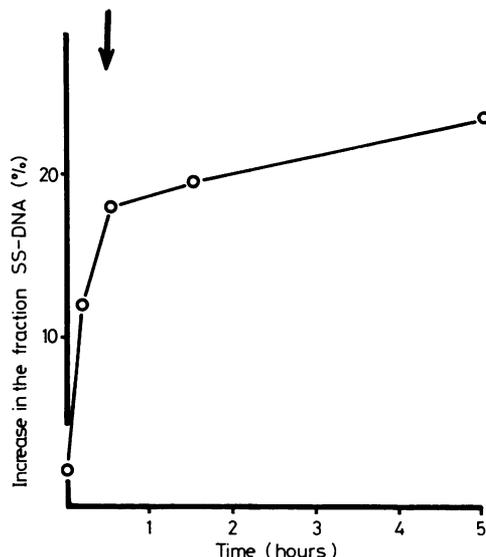


FIG 2 Induction and repair of DNA strand breaks in gossypol exposed human fibroblasts. Fibroblasts were incubated with gossypol (20 µg/ml) in PBS. After 30 minutes the medium was changed (arrow) and the culture was incubated in Eagle's MEM supplemented with 10% fetal calf serum. The increase in the fraction SS-DNA over background is given as the mean values of duplicate determinations.

were therefore carried out with 100-fold lower concentrations of gossypol in serum-free medium. Under these conditions gossypol was found to cause a weak but reproducible increase of SCE in two separate experiments (table 2). This effect is not likely to be the result of growth inhibition, since the lowest concentrations of gossypol used (40 and 100 ng/ml) did not retard the cell proliferation in these cultures, in spite of a clear increase of SCE (table 2).

TABLE 2 Sister chromatid exchanges in human lymphocytes treated with gossypol in serum-free medium for 1 hour at late G₁ phase.

	SCE/cell (mean ± SD)	No. of cells	Rate of cell proliferation†
Experiment 1			
Control (DMSO)	13.7 ± 3.3	40	58/42/0
Gossypol 40 ng/ml	18.1 ± 4.1*	40	51/49/0
Gossypol 100 ng/ml	17.6 ± 4.3*	40	46/54/0
MMS 50 µmol/l	20.7 ± 5.1*	20	46/53/1
Experiment 2			
Control (DMSO)	9.8 ± 1.7	20	62/38/0
Gossypol 400 ng/ml	14.3 ± 2.3*	20	88/12/0
Gossypol 4.0 µg/ml	13.7 ± 2.2*	20	82/18/0
MMS 100 µmol/l	21.4 ± 3.0*	20	81/19/0

*Student's *t* test, *p* < 0.001.

†The figures represent the proportion of 100 cells in first, second, and third division respectively.

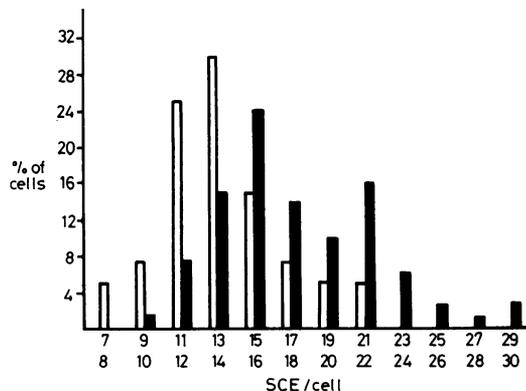


FIG 3 The distribution of SCEs in human lymphocytes exposed to gossypol for 1 hour in serum-free medium at late G₁ phase. Data from experiment 1, table 2. The control distribution (white columns) is based on 40 cells. The distribution in gossypol treated cells (black columns) is based on 80 cells, including both dose levels (40 and 100 ng/ml).

As shown in fig 3, gossypol treatment causes a marked shift of the SCE distribution towards higher SCE levels, and there is a substantial fraction of outlying cells with high SCE numbers in the gossypol treated cultures.

Discussion

Our results show that gossypol causes DNA strand breaks in cultured human skin fibroblasts. These breaks may arise either through a direct interaction with cellular DNA or indirectly through the generation of DNA damaging radicals. DNA strand scissions are found after exposure to chemical mutagens and carcinogens that bind to DNA, but may also be caused by non-mutagenic DNA binding chemical agents.^{18 19}

The finding that the number of DNA strand breaks was reduced to background when serum was added to the gossypol incubation indicates that the extent of protein binding of gossypol is high. This is in agreement with the very long half life of gossypol *in vivo* and the covalent binding of gossypol to different proteins.¹⁷ Protein binding must therefore be considered when evaluating *in vitro* results on the genotoxic effects of gossypol.

When human lymphocytes were incubated with low concentrations of gossypol in serum free medium, a clear increase in the number of SCEs was found. In a previous study, Tsui *et al*¹¹ found a slight increase of SCE in human lymphocytes exposed in serum-containing medium to gossypol at high concentrations which reduced cell proliferation.

Among other agents which cause DNA strand breaks, but only slight increase in SCE, are ionising radiation, bleomycin, dopamine, and oxygen radicals.²⁰⁻²³ These agents all appear to generate free radicals, which suggests that such a mechanism may also be involved in the genotoxic effect of gossypol.

Gossypol is readily oxidised to a quinonoid metabolite, gossypolone.⁷ Other quinones such as benzo(a)pyrene quinones and menadione may bind to DNA or cause DNA strand breaks or both.^{24 25} Dopamine, which is oxidised spontaneously to a semiquinone and subsequently to a quinone, was also recently shown to bind to DNA and to induce DNA strand breaks.²³ The genotoxic effects of these quinones may be the result of the formation of different types of radicals or adducts.^{23 24} Similarly, the genotoxic effects of gossypol may involve the oxidation of gossypol to a quinonoid DNA binding metabolite or the formation of a toxic and DNA damaging radical.

The results presented indicate that gossypol may induce genetic damage. Therefore, these results call for further investigation to determine if the inhibition of human spermatogenesis is the result of gossypol induced DNA damage.

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