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Effect of gossypol on the motility and metabolism of human spermatozoa

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Summary. Gossypol, a polycyclic compound isolated from cotton seeds, had a dose-dependent inhibitory effect on human sperm motility. The drug also inhibited powerfully fructolysis and glycolysis by human spermatozoa. Both lactate and CO_2 formation from the ^{14}C-labelled sugars was inhibited, and the prevention of CO_2 formation from [1-^{14}C]pyruvate and [2-^{14}C]pyruvate by gossypol indicated a direct effect on the tricarboxylic acid cycle. Repeated washing of the sperm cells after gossypol pretreatment failed to abolish the inhibitory effect on CO_2 production. The profound disturbances of the sperm energy metabolism induced by gossypol were also reflected by a striking fall of the sperm ATP content. Gossypol had little effect on glucose utilization by minces of human vaginal mucosa, indicating the specificity of gossypol.

Introduction

Gossypol (2,2′-binaphthalene-8,8′-dicarboxaldehyde-1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl), a phenolic compound containing two aldehyde groups, has been reported to exert a marked antifertility effect in males (National Coordinating Group on Male Infertility Agents, 1978). The mechanism of action of this compound is not known, but systemic administration of the drug to male rats results in early indications of disturbed spermatogenesis, although the onset of the antifertility effect is slow (2–4 weeks: National Coordinating Group on Male Infertility Agents, 1978). Electron microscopic examination of gossypol-treated rats revealed morphological changes in the spermatids, such as acrosomal fragmentation and damage to the mitochondrial sheath.

Evidence has also been presented indicating that gossypol does not preferentially accumulate in the testicular tissue: the concentration of the drug in the testis was not more than one third of that found in the liver (National Coordinating Group on Male Infertility Agents, 1978). Spermatids appear to be especially vulnerable to the compound, since no structural changes were observed in the Leydig cells in the gossypol-treated animals.

The effects of gossypol are, however, not restricted to different phases of spermatogenesis. It has been reported to inhibit several enzymes of the tricarboxylic acid cycle (Tso, Lee & Tso, 1982; Montamat *et al.*, 1982), LDH isoenzyme X (Lee, Moon, Yan & Chen, 1982), segments of electron transport chain (Tso & Lee, 1982), and glutathione S-transferase (Lee & Malling, 1981), and to uncouple respiratory chain and oxidative phosphorylation (Abou-Donia & Dieckert, 1974). The ATP concentration of rat epididymal spermatozoa correlates closely with the sperm motility (Ke & Tso, 1982).

Pösö, Wichmann, Jänne & Luukkainen (1980) reported that low concentrations of gossypol exert a direct effect on mature (motile) human spermatozoa as indicated by a striking decrease in sugar degradation and gradual loss of motility. We have now expanded these studies of the effects of gossypol on the metabolism of human spermatozoa.

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Materials and Methods

Semen samples. Semen was obtained from a local clinical laboratory performing semen analyses. Based on motility (50–80%) and morphology, apparently normal, pooled semen samples were first diluted (1 :1) with a Ringer solution containing 123 mM-NaCl, 5 mM-KCl, 1 mM-$MgSO_4$, 500 i.u. benzylpenicillin/ml and 0·2 mg streptomycin sulphate/ml and buffered with 37 mM-Tris–HCl, pH 8·0 (Murdoch & White, 1968; Eliasson, 1971). The spermatozoa were separated from the seminal plasma by low-speed centrifugation (75 ***g***, 10 min) at room temperature, washed twice with the Ringer solution and suspended in the same solution at a cell density of 50–100 × 10^6 cells/ml.

Chemicals. [U-^{14}C]Fructose (sp. act. 283 Ci/mmol), [U-^{14}C]glucose (sp. act. 333 mCi/mmol), [1-^{14}C]pyruvic acid, sodium salt (sp. act. 17·3 mCi/mmol) and [2-^{14}C]pyruvic acid, sodium salt (sp. act. 16·6 mCi/mmol) were all purchased from the Radiochemical Centre (Amersham, Bucks, U.K.). Other chemicals were of analytical grade of purity. Gossypol, a gift from Dr C. Chang (Population Council), was dissolved in absolute ethanol. The same amount of ethanol was used as the vehicle control. The purity of the gossypol preparation used was verified with both gas-liquid chromatography and mass spectrometry.

Determination of sperm motility. The spermatozoa were washed twice with the Ringer solution containing 0·5% human plasma albumin, and the washed cells were suspended in the same solution. The suspension was preincubated in test tubes for 15 min at 37°C. Thereafter, different concentrations of gossypol were added, and samples were taken at successive times for motility determination by microscopic observation. The sample was diluted with Ringer solution, 100 cells were counted and the proportion of motile cells was estimated. The person performing the counting procedure was unaware of the order of the samples.

Degradation of glucose and fructose. Washed spermatozoa (100 × 10^6 cells/ml) were preincubated in Ringer solution (pH 8·0) at 37°C with various additions (see 'Results') for 20 min unless otherwise indicated. Then 0·5 μCi [U-^{14}C]glucose (final concentration 0·2 mM) or 0·5 μCi [U-^{14}C]fructose (final conc. 0·2 mM) were added, the tubes were capped tightly, and the incubation was continued for 30 min with gentle shaking. The final incubation volume was 0·4 ml. The CO_2 evolved was collected in 0·1 ml Soluene (Packard Instrument Company) and counted for radioactivity. The reaction was halted by an addition of 0·5 ml 25% (w/v) trichloroacetic acid, and 0·2 ml of the protein-free supernatant solution was used for lactate determination by the method of Hoskins & Patterson (1968).

Formation of CO_2 from pyruvate. Washed spermatozoa (100 × 10^6 cells/ml) were preincubated at 37°C (pH 8·0) with additions (see 'Results') for 20 min. Then 0·5 μCi [1-^{14}C]pyruvate or [2-^{14}C]pyruvate (final conc. 0·1 mM) was added, and the incubation was continued for 30 min. The radioactivity liberated was measured as described above.

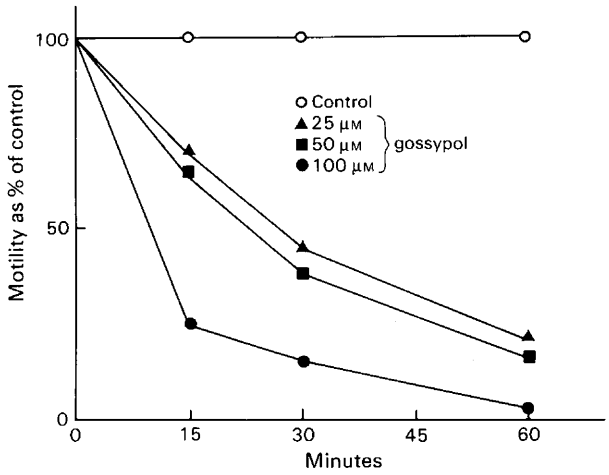
ATP content of the spermatozoa. Washed spermatozoa (100 × 10^6 cells/ml) were incubated at 37°C (pH 8·0) with additions (see 'Results') in a final volume of 0·4 ml. For ATP assay the sperm suspension was diluted with water to a final density of 2 × 10^6 cells/ml, and the cell ATP content was measured with a Lumac Celltester M1020 (Lumac Systems AG, Basel, Switzerland), according to the manufacturer's application bulletin. In this method, sperm cells are disrupted with a releasing reagent, and then ATP is determined using firefly luciferin-luciferase, which is specific to ATP. An internal ATP standard is used.

Degradation of glucose in vaginal mucosa minces. Minces of fresh human vaginal mucosa (50 mg in 0·4 ml Ringer solution) were preincubated for 20 min at 37°C (pH 8·0) with 0–100 μM-gossypol. Then 0·5 μCi [U-^{14}C]glucose (final conc. 0·025 mM) was added and the incubation was continued for 30 min. The degradation of glucose was followed as described for the spermatozoa.

Results

Effect of gossypol on the motility of human spermatozoa

The gradual immobilization of human spermatozoa in the presence of gossypol is demonstrated in Text-fig. 1. The spermatozoa were also rapidly immobilized in human cervical mucus preparations containing 50–100 μM-gossypol (results not shown).



Text-fig. 1. Effect of increasing gossypol concentrations on the motility of ejaculated human spermatozoa. Values are of a representative experiment but were similar in at least 2 other experiments.

Inhibition of sperm fructolysis by gossypol

When freshly ejaculated spermatozoa were exposed to increasing concentrations (5–50 μM) of gossypol, the degradation of radioactive fructose to lactate and CO_2 was markedly inhibited (Table 1). The effect was dose-dependent, and 50 μM-gossypol caused a total inhibition of the fructolytic activity. The degradation of [U-^{14}C]glucose to lactate and CO_2 was similarly inhibited, although low gossypol concentrations (5–10 μM) appeared to have little effect on the degradation (results not

Table 1. Effect of different gossypol concentrations on human sperm fructolysis

Gossypol (μM)	CO_2		[^{14}C]lactate	
	pmol/10^6 cells	(%)	pmol/10^6 cells	(%)
0	119·0 ± 25·3	(100)	649·9 ± 225·1	(100)
5	107·2 ± 25·3	(90)	477·7 ± 202·5	(90)
10	45·5 ± 17·1	(38)	142·4 ± 77·8	(22)
50	3·4 ± 2·1	(3)	13·6 ± 6·9	(2)

For experimental details see 'Materials and Methods'. The values are means (±s.d.) of 4 separate experiments.

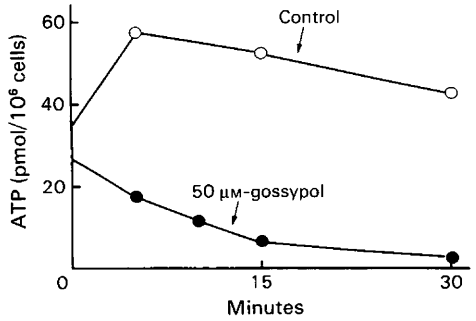
Table 2. Effect of gossypol on the formation of carbon dioxide from [1-^{14}C]pyruvate and [2-^{14}C]pyruvate by human spermatozoa

Gossypol (μM)	Formation of CO_2 (pmol/10^6 cells)			
	[1-^{14}C]pyruvate	(%)	[2-^{14}C]pyruvate	(%)
0	429·1 ± 188·1	(100)	28·0 ± 5·3	(100)
10	373·6 ± 149·7	(87)	24·4 ± 6·3	(87)
25	155·5 ± 108·1	(36)	4·4 ± 2·2	(16)
50	73·4 ± 87·5	(17)	1·0 ± 0·3	(4)

The spermatozoa were exposed to increasing concentrations of gossypol and incubated in the presence of [1-^{14}C]- or [2-^{14}C]pyruvate as described in 'Materials and Methods'. The values are means (±s.d.) obtained from 4 separate experiments.

shown). The results indicated that gossypol might inhibit some steps of the glycolytic pathway. To study whether the drug had any influence on the reactions of the tricarboxylic acid cycle, gossypol-treated spermatozoa were incubated in the presence of [1-^{14}C]pyruvate or [2-^{14}C]pyruvate and the formation of radioactive CO_2 from these substrates was measured (Table 2). A dose of 10 μM-gossypol appeared to have little effect on the degradation of either labelled compound, but higher drug concentrations (25 and 50 μM) inhibited profoundly the CO_2 evolution, especially from [2-^{14}C]pyruvate.

Text-figure 2 shows that 50 μM-gossypol depleted the sperm cells of ATP in 30 min, while the ATP content of the control cells remained virtually unchanged.



Text-fig. 2. Effect of gossypol on human sperm ATP content. Values are of a representative experiment but were similar in at least 2 other experiments.

Attachment of gossypol to sperm cells

Spermatozoa were incubated for 20 min in the presence of increasing (0–100 μM) gossypol concentrations. Then the gossypol-containing medium was removed and the cells were washed twice. The formation of CO_2 from glucose was determined in a gossypol-free medium. Table 3 shows that removal of gossypol by careful washing after the 20-min preincubation did not restore the glycolytic activity.

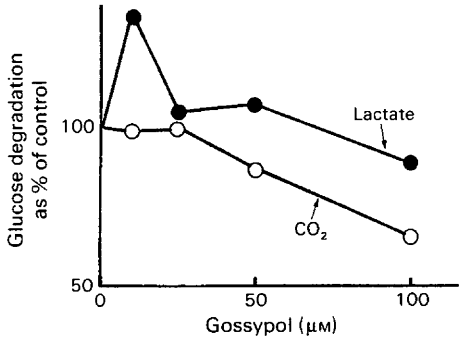
Table 3. Effect of repeated washings on glucose utilization of human spermatozoa exposed to gossypol

Gossypol (μM)	CO_2 (pmol/10⁶ cells)			
	Cells with gossypol	(%)	Washed cells	(%)
0	69·9	(100)	28·1	(100)
25	16·6	(24)	6·3	(23)
50	9·9	(15)	2·7	(10)
100	2·0	(3)	0·5	(3)

The spermatozoa were exposed to increasing concentrations of gossypol for 30 min. Then the formation of CO_2 from [^{14}C]glucose was measured in the presence of gossypol or, after washing the cells twice with Ringer solution, in a drug-free medium. Values are for a representative experiment but were similar in at least 2 other experiments.

Effect of gossypol on glucose utilization in human vaginal mucosa minces

Gossypol concentrations (up to 100 μM) that were totally inhibitory to sperm sugar degradation only moderately depressed energy-yielding sugar metabolism in vaginal minces (Text-fig. 3).



Text-fig. 3. Effect of gossypol on glucose degradation in minces of human vaginal mucosa. The preparations were treated as described in 'Materials and Methods'. The results represent the mean of 2 different mucosal samples.

Discussion

Ever since the antifertility effect of gossypol was recognized in China, a number of reports have described its effects on spermatogenesis and sperm metabolism. Chronic administration of gossypol to rats leads to mitochondrial and flagellar damage in testicular and epididymal spermatozoa (Hoffer, 1982; Oko & Hrudka, 1982) and to a decrease of sperm ATP content with a concomitant loss of motility (Ke & Tso, 1982). Mitochondrial involvement is also implicated by studies suggesting that gossypol may act as an uncoupler of mitochondrial oxidative phosphorylation (Abou-Donia & Dieckert, 1974), and inhibit lactate dehydrogenase X, an enzyme postulated to participate in a shuttle system transferring H^+ from cytosol to mitochondria (Gerez de Burgos, Burgos, Montamat, Moreno & Blanco, 1978).

The present results show that gossypol exerts a profound inhibitory effect on sperm sugar degradation *in vitro*. Glucose and fructose (Table 1) utilization was totally inhibited in the presence of 50 μM-gossypol, which is in good agreement with our earlier observation (Pösö *et al.*, 1980). Sperm CO_2 production was also inhibited when radioactive pyruvate was used as the substrate (Table 2), suggesting a direct effect of gossypol on the enzymes of the tricarboxylic acid cycle. The results of Tso & Lee (1981) indicate that gossypol inhibits the sperm respiration rate when succinate is used as the substrate, while a considerably weaker inhibition was observed with malate and pyruvate as substrates. Further, Tso *et al.* (1982) reported that of several sperm enzymes tested, isocitrate dehydrogenase, succinyl-CoA synthetase and fumarase were inhibited by gossypol at concentrations below 0·1 mM.

Our studies revealed that gossypol strongly inhibits (80–90%) the uptake of radioactive fructose, glucose and pyruvate into spermatozoa whose fructolysis was prevented by iodoacetate (results not shown). This makes it very difficult to distinguish a solely membrane effect from a combined membrane and intracellular action. It appears evident, however, that gossypol is able to traverse the cell membrane (Montamat *et al.*, 1982), and the differential effect of gossypol on [1-^{14}C]pyruvate and [2-^{14}C]pyruvate degradation (Table 2) indicates that processes other than mere uptake are affected by the drug; gossypol also inhibited fructolysis in cell-free extracts (results not shown). Nevertheless, the fact remains that gossypol profoundly disturbs the uptake of glucose (and of fructose), as well as that of pyruvate, into the spermatozoon.

Because of the block in energy production, the sperm ATP concentration decreased rapidly (Text-fig. 2), concomitantly with the loss of sperm motility (Text-fig. 1). The decrease of the ATP level may be caused both by the inhibition of glycolysis and the putative uncoupling of oxidative phosphorylation. The initial rise in the ATP content of the control cells is probably due to transfer of the cells from room temperature (during washing) to 37°C, which is the incubation temperature.

Hoffer (1982) indicated that the deleterious morphological effects of gossypol *in vivo* are specific to spermatozoa, since no changes were observed in the epididymal or vasal epithelium. The results of Text-fig. 3 show that gossypol is also relatively inert in human vaginal mucosa cells. The apparent specificity of gossypol towards spermatozoa could lead to gossypol-based vaginal contraceptives.

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