Mechanism of inhibition by gossypol of glycolysis and motility of monkey spermatozoa *in vitro*

D. T. Stephens, L. M. Critchlow and D. D. Hoskins

Oregon Regional Primate Research Center, 505 N.W. 185th Avenue, Beaverton, Oregon 97006, U.S.A.

Summary. Low levels of gossypol inhibited motility and anaerobic glycolysis of ejaculated rhesus monkey spermatozoa. Inhibition (50%) of both occurred at a gossypol:sperm ratio of 8 nmol/10⁸ spermatozoa, and complete inhibition of both occurred at a ratio of 75 nmol/10⁸ spermatozoa. Determination and comparison of the levels of glycolytic intermediates in intact spermatozoa, incubated with and without gossypol, indicated that the only site of glycolytic inhibition was lactate dehydrogenase-X (EC 1.1.1.27). At gossypol:sperm ratios above 6 nmol/10⁸ spermatozoa, gossypol also decreased the concentrations of the adenine nucleotides, ATP, ADP and AMP, and this is most probably the basis for its toxic effect on spermatozoa.

Introduction

Although gossypol has been shown to be an orally active contraceptive agent in a number of species (Chang, Zhi & Saksena, 1980; Hadley, Lin & Dym, 1981; Hahn, Rusticus, Probst, Homm & Johnson, 1981; Waller, Fong, Cordell & Soejarto, 1981), including man (National Coordinating Group, 1978; Qian *et al.*, 1980; Liu, 1981), reservations about its use in man have arisen because of reports of possible toxicity, side effects, and incomplete reversibility of spermatogenesis inhibition after drug withdrawal (Prasad & Diczfalussy, 1982). Because gossypol inhibits sperm motility both *in vitro* (Pösö, Wichmann, Jänne & Luukkainen, 1980; Kalla & Vasudev, 1980; Waller, Zaneveld & Fong, 1980; Ridley & Blasco, 1981; Tso & Lee, 1982) and *in vivo* (Cameron, Waller & Zaneveld, 1982), it has been suggested that gossypol has potential as a topical, vaginal contraceptive (Pösö *et al.*, 1980; Cameron *et al.*, 1982; Tso & Lee, 1982), an idea supported by the exceedingly small amounts required to inhibit sperm motility (Pösö *et al.*, 1980; Ridley & Blasco, 1981). At present, however, little is known of the biochemical mechanisms by which gossypol inhibits motility. One brief report has mentioned its ability to inhibit human sperm glycolysis (Pösö *et al.*, 1980), and there are two very brief descriptions of its ability to inhibit solubilized human sperm lactate dehydrogenase-X (LDH-X) (EC 1.1.1.27) (Lee & Malling, 1981; Montamat, Burgos, Burgos, Rovai & Blanco, 1982).

The purpose of the present study was to examine in more detail the mechanism by which gossypol inhibits sperm motility and glycolysis in a non-human primate, the rhesus macaque (*Macaca mulatta*).

Materials and Methods

Semen was collected from 10 rhesus macaques (*M. mulatta*) at 4-day intervals by electroejaculation. Spermatozoa were isolated and washed in Ca²⁺-free Krebs–Ringer solution containing 50 mM-Tris (hydroxymethyl) aminomethane–HCl (Tris–HCl), pH 7.4 (KRT), as described elsewhere (Hoskins

& Patterson, 1968). Rates of glycolysis were determined through measurement of the formation of [^{14}C]lactate from uniformly labelled [^{14}C]glucose (Hoskins & Patterson, 1968; Hoskins & Stephens, 1971). Each reaction tube contained the following in a 400- μl total reaction volume: 50 mM-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) buffer, pH 7.5; 83 mM-NaCl; 2.2 μM -2-*N*-heptyl-4-hydroxyquinoline *N*-oxide; 7.5 mM-unlabelled glucose; 2.5 μCi [U- ^{14}C]glucose/ml; 1% dimethyl sulphoxide (DMSO); gossypol-acetic acid (0–120 μM); and spermatozoa (0–2.5 $\times 10^8$ cells/ml). This amount of 2-*N*-heptyl-4-hydroxyquinoline *N*-oxide is sufficient to abolish completely the respiration of rhesus spermatozoa (Hoskins & Patterson, 1968). Assays were terminated after 1 h by the addition of 100 μl 25% trichloroacetic acid, and the [^{14}C]lactate formation was determined (Hoskins & Patterson, 1968).

Glycolytic intermediates were assayed fluorometrically in perchloric acid extracts of sperm suspensions as described elsewhere (Hoskins, Stephens & Casillas, 1971). Adenine nucleotides in trichloroacetic acid extracts were assayed fluorometrically (Greengard, 1962) or by liquid scintillation spectrometry with luciferase (Hoskins, 1973). Motility, evaluated by 4 observers, was rated on a scale of 1 to 10. Gossypol-acetic acid was freshly prepared by dissolving in DMSO and then diluting with water to yield the desired final concentration of gossypol-acetic acid and a DMSO concentration of 1%. All control incubation tubes contained 1% DMSO.

Gossypol-acetic acid was purchased from the Sigma Chemical Company (St Louis, Missouri, U.S.A.). All other chemicals and radiochemicals used were of the highest purity available.

Results

Gossypol inhibited the motility of ejaculated monkey spermatozoa in a concentration-dependent manner (Text-fig. 1). At concentrations of 15 μM or less, little inhibition occurred. A progressive loss of motility was seen in the range of 20–50 μM , and complete inhibition occurred at a concentration of 75 μM . The inhibition was apparently accompanied by an irreversible binding of gossypol to spermatozoa because motility was not restored after removal of the gossypol by washing and, after washing, the yellow gossypol pigment was associated with the sedimented spermatozoa. A 50% inhibition of motility occurred at a gossypol:sperm ratio of approximately 8 nmol/ 10^8 spermatozoa (Text-fig. 1). This effect of gossypol on anaerobic sperm motility was nearly identical to the effect on anaerobic glycolysis (Text-fig. 2), i.e. glycolysis measured in the presence of the electron transport chain inhibitor 2-*N*-heptyl-4-hydroxyquinoline *N*-oxide. This inhibitor was included to inhibit respiration and thereby to make the spermatozoa dependent on glycolysis.

In numerous initial experiments, we observed that the extent of inhibition effected by a given concentration of gossypol varied greatly. It subsequently became clear that only when the results were expressed in terms of a gossypol:sperm ratio, rather than the gossypol concentration, did the glycolytic inhibition data describe, as shown in Text-fig. 2, an essentially smooth curve falling from 100% to nearly 0% of control values as the ratio was increased from 0 to 50 nmol/ 10^8 spermatozoa. There was minimal scatter in the distribution of the datum points: in 7 experiments the gossypol concentration ranged from 1 to 120 μM and the sperm concentration from 0.1 to 5×10^8 cells/ml. Text-figure 2 also shows that 50% inhibition of glycolysis occurred at a gossypol:sperm ratio of 8 nmol/ 10^8 spermatozoa, a value identical to that observed for the inhibition of motility.

To determine the enzymic site (or sites) of inhibition in the glycolytic sequence, all glycolytic intermediates in the presence and absence of gossypol were measured and the so-called 'cross-over points' were identified. We anticipated that the substrate(s) of the glycolytic enzymes affected by gossypol would change in a direction opposite to the direction of change in the glycolytic rate (Hoskins *et al.*, 1971; Newsholme & Start, 1973). Since gossypol lowers the glycolytic rate, the substrate of the inhibited sperm enzyme will increase. The data in Table 1 show (1) that 1% DMSO did not significantly alter the levels of intermediates from values in control suspensions, (2) only the

Text-fig. 1. Inhibition of monkey sperm motility by gossypol. Motility was evaluated on a scale of 1 to 10 by 4 observers; the scores were then averaged. Incubation conditions were those used for the glycolytic incubation (see 'Methods'), but without labelled glucose. Each tube contained DMSO at 1% and 1.6×10^8 spermatozoa in 400 μ l. The triangle indicates a control without DMSO.

Text-fig. 2. Effect of gossypol:sperm ratio on glycolysis of monkey spermatozoa. The data represent combined results from 7 experiments. The triangle represents a control without DMSO. For procedural details, see 'Methods'.

level of pyruvate increased significantly in the presence of gossypol, and (3) the levels of three intermediates, fructose-1,6-diphosphate, dihydroxyacetonephosphate and possibly ($P < 0.1$) glyceraldehyde-3-phosphate, were decreased.

The inhibition of sperm glycolysis by gossypol should be accompanied by a decrease in the rate of ATP formation, the energy source for sperm motility. In another series of experiments, we examined the effect of gossypol on adenine nucleotide levels of intact spermatozoa. The ATP, ADP, and AMP levels in cells incubated in the presence of 2–40 nmol gossypol/ 10^8 spermatozoa in 1% DMSO were measured and compared to values obtained with control sperm suspensions incubated in the presence or absence of 1% DMSO. The size of the total nucleotide pool (ATP plus ADP plus AMP) and the energy charge was also calculated. The data in Table 2 show, as was the

Table 1. Effects of gossypol on levels of glycolytic intermediates in monkey spermatozoa

Intermediate	Intermediate content (nmol/10 ⁸ spermatozoa)		
	Control	+1% DMSO	+Gossypol (8 nmol/10 ⁸ sperm.)
Glucose-6-phosphate	2.2 ± 0.5	2.3 ± 0.5	2.1 ± 0.2
Fructose-6-phosphate	1.4 ± 0.3	1.3 ± 0.3	0.8 ± 0.1
Fructose-1,6-diphosphate	15.6 ± 2.6	18.2 ± 3.4	9.3 ± 2.7*
Dihydroxyacetone phosphate	7.7 ± 0.8	6.7 ± 0.6	4.4 ± 0.7**
Glyceraldehyde-3-phosphate	9.7 ± 1.1	8.0 ± 1.3	6.2 ± 1.4***
3-Phosphoglycerate	0.13 ± 0.01	0.16 ± 0.02	0.17 ± 0.02
2-Phosphoglycerate	0.07 ± 0.02	0.06 ± 0.01	0.09 ± 0.03
Phosphoenolpyruvate	0.09 ± 0.02	0.10 ± 0.02	0.12 ± 0.05
Pyruvate	0.25 ± 0.04	0.24 ± 0.04	0.37 ± 0.03*

Values are means ± s.e.m. for 4–8 replicate experiments.

Compared to DMSO control: **P* < 0.02; ***P* < 0.05; ****P* < 0.1.

Table 2. Effect of gossypol:sperm ratio on adenine nucleotide content of monkey spermatozoa

Gossypol:sperm ratio (nmol/10 ⁸ spermatozoa)	Adenine nucleotide content (nmol/10 ⁸ spermatozoa)				Energy charge
	ATP	ADP	AMP	Total pool	
0 (control)	16 ± 1 (13)	7 ± 1 (7)	7 ± 2 (7)	29 ± 2 (7)	0.67
0 (+ 1% DMSO)	16 ± 1 (9)	6 ± 1 (4)	5 ± 2 (5)	28 ± 1 (4)	0.68
2	16 ± 3 (6)	7 ± 1 (4)	7 ± 3 (4)	31 ± 3 (4)	0.63
4	16 ± 2 (7)	7 ± 1 (5)	6 ± 2 (5)	30 ± 1 (5)	0.65
6	15 ± 2 (7)	5 ± 1 (7)	5 ± 2 (7)	25 ± 1 (7)	0.70
8	12 ± 2 (8)	5 ± 1 (8)	4 ± 1 (8)	21 ± 3 (8)	0.69
20	9 ± 2 (8)	2 ± 1 (7)	3 ± 1 (7)	16 ± 3 (7)	0.34
40	2 ± 1 (11)	0 (8)	0 (8)	3 ± 1 (8)	—

Values are means ± s.e.m. for the numbers of replicate experiments indicated in parentheses.

case for glycolytic intermediate levels, that the solvent, 1% DMSO, had no significant effect on the level of adenine nucleotides. They further show that gossypol, at a ratio greater than 8 nmol/10⁸ spermatozoa, significantly decreased the intracellular ATP content and that nearly complete depletion occurred at the highest level (40 nmol/10⁸ spermatozoa). Significantly, the intracellular content of ADP and AMP also dropped at the higher gossypol ratios, particularly at 40 nmol/10⁸ spermatozoa. The end result was a marked reduction in the total adenine nucleotide pool from 30 to 3 nmol nucleotide/10⁸ spermatozoa. At the gossypol ratio used to determine the site of glycolytic inhibition (8 nmol/10⁸ spermatozoa, Table 1), the levels of ATP, ADP and AMP were all beginning to drop. At the highest ratio tested, 40 nmol/10⁸ spermatozoa, ADP and AMP were undetectable and the ATP level was reduced to only 12% of control values. In a parallel experiment, gossypol at the highest level present in sample extracts had no effect on the determination of standard solutions of ATP, ADP or AMP. These data show that gossypol depleted sperm ATP, ADP and AMP levels, an effect that was particularly severe above a gossypol:sperm ratio of 20 nmol/10⁸ spermatozoa.

Discussion

In this study, we have shown that gossypol markedly inhibits the motility of rhesus spermatozoa, an effect similar to that reported for human (Pösö *et al.*, 1980; Waller *et al.*, 1980; Kalla & Vasudev, 1980; Ridley & Blasco, 1981), boar (Tso & Lee, 1982), and *Macaca arctoides* (Cameron *et al.*, 1982).

spermatozoa. That this inhibition of motility, as well as glycolysis, results from the binding of gossypol to the spermatozoa in an irreversible manner is suggested by the visible association of gossypol with sedimented spermatozoa after washing; by the inability to restore motility to gossypol-treated spermatozoa by washing; and by the dependency of the inhibition of glycolysis and motility on the ratio of gossypol to sperm rather than on the absolute gossypol concentration. This observation may well explain the variability in the effects of gossypol in previously reported studies (Pösö *et al.*, 1980; Kalla & Vasudev, 1980; Ridley & Blasco, 1981; Tso & Lee, 1982) since the effect of a given micromolar concentration of gossypol will, of course, vary greatly depending on the sperm concentration.

The unique presence of LDH-X in mammalian spermatozoa and spermatogenic cells has been postulated as the basis of the specific effect, at low levels, of orally ingested gossypol on the reproductive competency of the male (Lee & Malling, 1981). This postulate has been based, however, solely on in-vitro enzyme assays in which inhibition was observed in the presence of gossypol (Lee & Malling, 1981; Montamat *et al.*, 1982). In view of the high reactivity of the gossypol molecule (Lyman, Baliga & Slay, 1959; Berardi & Goldblatt, 1980), such in-vitro inhibition data are difficult to interpret. Our results (Table 1) suggest that in intact spermatozoa gossypol inhibits the glycolytic sequence at the site of LDH-X. This follows because LDH-X is the predominant (> 80%) isoenzyme in the cytosol of mature spermatozoa (Machado de Domenech, Domenech, Aoki & Blanco, 1972; Storey & Kayne, 1977).

This inhibition appears to have both direct and indirect effects. One direct effect is a lowering of the intracellular level of ATP due to the diminished rate of glycolytic ATP synthesis. Since respiratory replenishment of ATP levels was blocked in our experiments by the electron transport chain inhibitor 2-*N*-heptyl-4-hydroxyquinoline *N*-oxide, the high rate of ATP utilization to maintain motility caused a drop in the level of ATP. In addition, with higher levels of gossypol, a marked decrease in the total adenine nucleotide pool occurred (Table 2). This may well have reflected an indirect gossypol effect, in that the reduction probably was due to a protective response of spermatozoa to decreases in the energy charge. This response does occur in human spermatozoa (Chulavatnatol & Haesungcharern, 1977). The net effect is an attempt by the spermatozoa to compensate for a drop in the cellular energy charge by a reduction in the total adenine nucleotide pool size. However, this compensation mechanism can only protect the energy charge from transient perturbation of the balance of ATP synthesis and utilization (Chapman & Atkinson, 1973). The severe inhibition of glycolytic ATP synthesis by high levels of gossypol appears to be more than can be compensated for by this mechanism, and results in a 'lethal' reduction in the adenine nucleotide content of the spermatozoa since both motility and glycolysis require ATP.

We found evidence for a second indirect effect of gossypol on sperm glycolysis in our experiment on altered levels of the glycolytic intermediates in the presence of gossypol (Table 1). The steady-state levels of fructose-1,6-diphosphate, dihydroxyacetone phosphate and possibly glyceraldehyde-3-phosphate are reduced by gossypol. Since all three are essentially in equilibrium, their reduction would appear to indicate an activation of glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12). However, since LDH is inhibited by gossypol, one would expect an accumulation of intermediates between glyceraldehyde 3-phosphate dehydrogenase and LDH. Since this is not observed, glyceraldehyde 3-phosphate dehydrogenase is presumably not activated. Another explanation for the decrease in these three intermediates is an activation of α -glycerolphosphate dehydrogenase (EC 1.1.1.8). Such an activation may occur due to mass-action effects of the NADH accumulation caused by the primary effect of gossypol—an inhibition of LDH-X. Alternatively, the lowered ATP levels may be insufficient to allow phosphorylation of fructose-6-phosphate by phosphofructokinase (i.e. 'priming' of glycolysis) and thereby results in the lowering of the levels of the products of phosphofructokinase.

In summary, gossypol appears to bind irreversibly to the intact ejaculated spermatozoa of monkeys and to inhibit sperm glycolysis by virtue of its inhibition of a sperm-specific enzyme, LDH-X. The inhibition of this enzyme depletes the sperm adenine nucleotide pool and thereby results in diminished motility and eventual cell death.

The work described in this article, Publication No. 1269 of the Oregon Regional Primate Research Center, was supported in part by NICHD grant HD-11982 and NIH grant RR-00163. We thank Jean McKinnon for her assistance in the preparation of the manuscript.

References

- Berardi, L.L. & Goldblatt, L.A.** (1980) Gossypol. In *Toxic Constituents of Plant Foodstuffs*, 2nd edn, pp. 183–237. Ed. I. E. Liener. Academic Press, New York.
- Cameron, S.M., Waller, D.P. & Zaneveld, L.J.D.** (1982) Vaginal spermicidal activity of gossypol in the *Macaca arctoides*. *Fert. Steril.* **37**, 273–274.
- Chang, M.C., Zhi, P.G. & Saksena, S.K.** (1980) Effects of gossypol on the fertility of male rats, hamsters, and rabbits. *Contraception* **21**, 461–469.
- Chapman, A.G. & Atkinson, D.E.** (1973) Stabilization of adenylate energy charge by the adenylate deaminase reaction. *J. biol. Chem.* **248**, 8309–8312.
- Chulavatnatol, M. & Haesungcharern, A.** (1977) Stabilization of adenylate energy charge and its relation to human sperm motility. *J. biol. Chem.* **252**, 8088–8091.
- Greengard, P.** (1962) Cited in *Fluorescence Assay*, p. 332. Ed. S. Udenfriend. Academic Press, New York.
- Hadley, M.A., Lin, C.Y. & Dym, M.** (1981) Effects of gossypol on the reproductive system of male rats. *J. Androl.* **2**, 190–194.
- Hahn, D.W., Rusticus, A., Probst, A., Homm, R. & Johnson, A.N.** (1981) Antifertility and endocrine activities of gossypol in rodents. *Contraception* **24**, 97–99.
- Hoskins, D.D.** (1973) Adenine nucleotide mediation of fructolysis and motility in bovine epididymal spermatozoa. *J. biol. Chem.* **248**, 1135–1140.
- Hoskins, D.D. & Patterson, D.L.** (1968) Metabolism of rhesus monkey spermatozoa. *J. Reprod. Fert.* **16**, 183–195.
- Hoskins, D.D. & Stephens, D.T.** (1971) Filipin-induced inhibition of fructolysis and motility in monkey spermatozoa. *Biol. Reprod.* **5**, 330–335.
- Hoskins, D.D., Stephens, D.T. & Casillas, E.R.** (1971) Enzymic control of fructolysis in primate spermatozoa. *Biochim. Biophys. Acta* **237**, 227–238.
- Kalla, N.R. & Vasudev, M.** (1980) Studies on the male antifertility agent gossypol acetic acid. I. In-vitro studies on the effect of gossypol acetic acid in human spermatozoa. *IRCS Med. Sci. Biochem.* **8**, 375–379.
- Lee, C.Y. & Malling, H.Y.** (1981) Selective inhibition of sperm-specific lactate dehydrogenase X by the antifertility agent, gossypol. *Fedn Proc. Fedn Am. Soc. exp. Biol.* **40**, 718, Abstr.
- Liu, G.Z.** (1981) Clinical study of gossypol as a male contraceptive. *Reproduction* **5**, 189–193.
- Lyman, C.M., Baliga, B.P. & Slay, M.W.** (1959) Reactions of proteins with gossypol. *Archs Biochem. Biophys.* **84**, 486–497.
- Machado de Domenech, E., Domenech, C.E., Aoki, A. & Blanco, A.** (1972) Association of the testicular lactate dehydrogenase isozyme with a special type of mitochondria. *Biol. Reprod.* **6**, 136–147.
- Montamat, E.E., Burgos, C., Burgos, N.M.G., Rovai, L.E. & Blanco, A.** (1982) Inhibitory action of gossypol on enzymes and growth of *Trypanosoma cruzi*. *Science, N.Y.* **218**, 288–289.
- National Coordinating Group on Male Antifertility Agents** (1978) Gossypol. A new antifertility agent for males. *Chin. Med. J. (English edn)* **4**, 417–428.
- Newsholme, E.A. & Start, C.** (1973) *Regulation in Metabolism*, p. 16. Wiley, New York.
- Pösö, H., Wichmann, K., Jänne, J. & Luukkainen, T.** (1980) Gossypol: a powerful inhibitor of human spermatozoal metabolism. *Lancet* **i**, 885–886.
- Prasad, M.R.N. & Diczfalussy, E.** (1982) Gossypol. *Int. J. Androl., Suppl.* **5**, 53–70.
- Qian, S.Z., Hu, J.H., Ho, L.X., Sun, M.X., Huang, Y.Z. & Fang, J.H.** (1980) The first clinical trial of gossypol on male antifertility. *Proc. 1st Conf. Clin. Pharmacol. Therapeut. Wuhan*, pp. 489–492. Ed. P. Turner. Macmillan, London.
- Ridley, A.J. & Blasco, L.** (1981) Testosterone and gossypol effects on human sperm motility. *Fert. Steril.* **36**, 638–642.
- Storey, B.T. & Kayne, F.J.** (1977) Energy metabolism of spermatozoa. VI. Direct intramitochondrial lactate oxidation by rabbit sperm mitochondria. *Biol. Reprod.* **16**, 549–556.
- Tso, W.-W. & Lee, C.-S.** (1982) Cottonseed oil as a vaginal contraceptive. *Arch. Androl.* **8**, 11–14.
- Waller, D.P., Zaneveld, L.J.D. & Fong, H.H.S.** (1980) In-vitro spermicidal activity of gossypol. *Contraception* **22**, 183–186.
- Waller, D.P., Fong, H.H.S., Cordell, G.F. & Soejarto, D.D.** (1981) Antifertility effects of gossypol and its impurities on male hamsters. *Contraception* **23**, 653–658.

Received 14 January 1983