Differential effects of (+)- and (–)-gossypol enantiomers on LDH-C₄ activity of hamster spermatogenic epithelium *in vitro*

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Summary. Tubular fragments (spermatogenic epithelium) from immature hamsters were isolated and cultured with low doses of (+)- and (–)-gossypol enantiomers. The activity of lactate dehydrogenase isoenzyme LDH-C₄ was estimated as a marker for spermatogenic cell development and α -ketoisovalerate was used as the substrate. In the absence of gossypol, the specific activity of LDH-C₄ in the tubular fragments was increased 40% during incubation for 48 h. This developmental increase was suppressed by gossypol. The specific activity of LDH-C₄ in the tubular fragments was lowered by gossypol, after 48 h of culture in the presence of low doses of racemic gossypol (1–4 μ M) and 1% fetal calf serum. In this in-vitro system the (–)-enantiomer but not the (+)-enantiomer of gossypol affected the LDH-C₄ activity. This is in agreement with other reports showing that only the (–)-enantiomer induces infertility. The observed action of gossypol on LDH-C₄ activity in the tubular fragments may reflect gossypol-induced degeneration of spermatogenic cells. The present in-vitro system can be used to estimate the actions of gossypol derivatives, other investigational antifertility agents, and toxic agents on the spermatogenic epithelium.

Keywords: gossypol enantiomers; spermatogenic epithelium; LDH-C₄; hamster

Introduction

Gossypol, a yellow polyphenolic compound extracted from cottonseed, is a racemic mixture of two enantiomers. The rotation about the C–C bond linking the two naphthyl rings is restricted, which results in (+)- and (–)-enantiomeric forms (Sampath & Balaram, 1986). Racemic gossypol is an effective antifertility agent in male rats and hamsters, and also in men (Prasad & Diczfalusy, 1982), but only the (–)-enantiomer of gossypol actively inhibits male fertility (Lindberg *et al.*, 1987).

During gossypol treatment of rats, rapid signs of damage include tail and head alterations in epididymal spermatozoa, and sperm immotility (Hoffer, 1982; Baccetti *et al.*, 1986). In the testis from rats treated for 2 weeks with gossypol, mitochondrial swelling and cristae disorganization in the mitochondrial sheath of step 18 and 19 spermatids has been observed (Hoffer, 1983; Baccetti *et al.*, 1986). Prolonged gossypol treatment resulted in degeneration of early spermatids and late-pachytene spermatocytes (National Coordinating Group of Male Contraceptive Studies, 1981). Studies on other cell types in the testis indicated that Sertoli cells were also damaged by gossypol treatment. These Sertoli cells exhibited large vacuoles and other signs of degeneration, but the mitochondria were not affected. The effects on Sertoli cells were not seen until after the appearance of the effects on late spermatids and sperm motility (Hoffer, 1983).

The mechanism by which gossypol affects the spermatogenic cells and spermatozoa has not been established. Several possible biochemical mechanisms of gossypol action have been described. These include effects on mitochondrial electron transport and oxidative phosphorylation, and inhibition of a number of enzymes involved in energy metabolism (Adeyemo *et al.*, 1982; Tso &

Lee, 1982a; Stephens *et al.*, 1983; Kim & Waller, 1984). Much attention has been paid to possible inhibition of the lactate dehydrogenase isoenzyme LDH-C₄ (Lee *et al.*, 1982; Tso & Lee, 1982b; Morris *et al.*, 1986; Whaley *et al.*, 1986). LDH-C₄ is composed of male germ cell-specific C subunits (Zinkham, 1968), and inhibition of this isoenzyme would specifically interfere with the metabolism of spermatogenic cells and spermatozoa. In-vivo administration of gossypol to rats can result in a decreased specific activity of testicular LDH-C₄ (U/mg protein), but it is not clear whether this decrease reflects the elimination of spermatogenic cells or direct inhibition of the enzyme (Olgati *et al.*, 1984a).

The aim of the present experiments was to develop an in-vitro system to study the effects of gossypol enantiomers and derivatives on the spermatogenic epithelium. Hamsters were used, mainly because hamsters have also been used in gossypol screening programmes to estimate the effects of gossypol and derivatives on spermatogenesis and fertility. Furthermore, hamsters are more sensitive to the antifertility effect of gossypol than are rats (Chang *et al.*, 1980).

Materials and Methods

Isolation and incubation of spermatogenic epithelium from hamsters. Immature 25–26-day-old hamsters (*Mesocricetus auratus*) were killed by cervical dislocation and the testes were removed. Tubular fragments were isolated by treatment of 6 decapsulated testes with 10 mg collagenase (CLS, Worthington Biochemical Corporation, Freehold, NJ, U.S.A.) in 20 ml phosphate-buffered saline (PBS), supplemented with 6 mM-L-lactate and 5.6 mM-glucose, for 30 min at 32°C in a shaking waterbath (120 cycles/min). The method used was the same, and yielded comparable results, as the method used to isolate tubular fragments from rat testes (Jutte *et al.*, 1982). The tubular fragments contained Sertoli cells and spermatogenic cells, and this preparation was also referred to as spermatogenic epithelium. In this preparation, the tubular wall had been largely removed, but a limited number of peritubular cells was still present.

The tubular fragments were incubated in Eagle's minimum essential medium (MEM) containing Earle's salts and 25 mM-Hepes (Gibco, Paisley, U.K.) supplemented with antibiotics (Grootegoed *et al.*, 1985), L-glutamine (292 mg/l) and 1% fetal calf serum (FCS). The incubations were performed for 48 h at 32°C under an atmosphere of 5% CO₂ in air, in a final volume of 2.5 ml in plastic 12 well plates (Costar, Broadway, Cambridge, MA, U.S.A.; approximately 0.9 mg protein/well with a growth area of 3.8 cm²).

Gossypol. Gossypol acetic acid (World Health Organization standard preparation) was dissolved in ethanol (stock solution of 1 mM) and stored at –20°C in the dark. The pure phenols (+)- and (–)-gossypol were obtained from S. A. Matlin (Department of Chemistry, The City University, London, U.K.), and were dissolved in ethanol shortly before use. A volume of 10 µl gossypol solution was added to 2.5 ml incubation medium. The maximum final concentration of ethanol in the incubations was 0.4% (v/v), and the same amount of ethanol was also added to the control incubations.

Estimation of LDH-C₄ activity. Tubular fragments of 4 wells were pooled and centrifuged (5 min, 600 g). The medium was discarded and the tubular fragments were sonicated in 1.6 ml water for 5 sec at 7 µm (MSE 150 Watt Ultrasonic disintegrator, 20 kHz), and frozen. After thawing and centrifugation (15 min, 10 000 g), 100–150 µl supernatant were used for estimation of the activity of the lactate dehydrogenase isoenzyme LDH-C₄, by measuring the oxidation of NADH at 340 nm using a Gilford model 2400 spectrophotometer (Blanco *et al.*, 1976). The enzyme assay mixture (final volume of 1 ml) contained 0.145 mM-NADH, 4.5 mM-EDTA, and different concentrations of the substrates, in 0.45 M-triethanolamine-HCl buffer (pH 7.6). Initial velocities were recorded for 2 min (ΔE was 0.02–0.03 per min). The results were expressed as international units (U) of enzyme activity per mg protein. LDH-C₄ from different species is reported to act specifically on a number of 2-oxo- and 2-hydroxy-acids (Blanco *et al.*, 1976; Coronel *et al.*, 1983). Different substrates for the enzymic reaction of LDH-C₄ were tested to find the most suitable substrate for the estimation of hamster LDH-C₄. The substrates tested were 2-oxobutanoate (α -ketobutyrate), 2-oxo-3-methylbutanoate (α -keto-isovalerate) and 2-oxo-4-methyl-pentanoate (α -ketoisocaproate) (Sigma Chemical Company, St Louis, MO, U.S.A.).

Estimation of cellular protein. The amount of cellular protein of the tubular fragments was estimated according to Lowry *et al.* (1951), using bovine serum albumin as standard.

Flow cytometric analysis. The cellular composition of the tubular fragments was estimated by DNA flow cytometry. This method yields information on the distribution of cells according to their DNA contents (the haploid spermatids contain a 1C amount of DNA; Sertoli cells, spermatogonia and secondary spermatocytes contain a 2C amount of DNA; primary spermatocytes contain a 4C amount of DNA). The DNA flow cytometric analysis was carried out essentially as described by Vindeløv *et al.* (1983a, b, c), as follows. Tubular fragments were centrifuged, the supernatant was discarded and the cells were resuspended in storage buffer (250 mM-sucrose, 40 mM-trisodium-citrate.2H₂O and 50 ml dimethylsulphoxide/l; pH 7.6). At this point, the preparations were frozen and stored at –80°C. Subsequently, the nuclei were prepared by trypsin treatment (30 mg/l; Worthington) for 10 min at room

temperature, in citrate-Tris buffer (3.4 mM-trisodiumcitrate.2H₂O, 0.5 M-Tris, pH 7.6) containing Nonidet P-40 (0.1%, v/v) as a detergent and spermine tetrachloride (1.5 mM) for stabilizing the nuclei. After this treatment, trypsin inhibitor (0.5 g/l; Sigma) and ribonuclease A (100 mg/l; Boehringer, Mannheim, F.R.G.), were added and the nuclei were incubated for another 10 min at room temperature. The final preparation of nuclei was stained with propidium iodide (416 mg/l) in the citrate-Tris buffer containing 4.5 mM-spermine tetrahydrochloride. The nuclei were filtered through a 30 µm nylon mesh and analysed using a FACS II flow cytometer (Becton Dickinson, Sunnyvale, CA, U.S.A.).

Results

Substrate specificity of hamster LDH-C₄

The reaction of the tubular homogenates with α -ketobutyrate showed the highest initial velocities, but the maximal initial velocity ($V_{\max}$) was not reached at substrate concentrations up to 6 mM (Fig. 1a). For evaluation of the enzyme kinetics, double-reciprocal (Lineweaver-Burk) plots were constructed. The Lineweaver-Burk plot for α -ketobutyrate did not yield a straight line (Fig. 1a), which indicates that this enzyme reaction deviates from Michaelis-Menten behaviour. A possible explanation is that more than one enzyme from the tubular fragments is reacting with this substrate. For this reason, α -ketobutyrate is not suitable as a substrate for the estimation of hamster LDH-C₄ activity. α -Ketoisovalerate gave somewhat lower initial velocities than did α -ketobutyrate, but the reaction could be better described according to the Michaelis-Menten equation (Fig. 1b), and a straight line was obtained in the Lineweaver-Burk plot for substrate concentrations up to 0.8 mM ($r = 0.995$) (Fig. 1b). Using the substrate α -ketoisocaproate, the reaction was inhibited at high substrate concentrations (Fig. 1c). The velocities obtained at low α -ketoisocaproate concentrations can be used to estimate K_m and $V_{\max}$ (Fig. 1c), but the low initial velocities and the sharp decline of enzyme activity at higher substrate concentrations limits the suitability of this substrate. In the experiments described below α -ketoisovalerate (1.2 mM) was used as substrate for the estimation of LDH-C₄ activities.

Correlation between LDH-C₄ activity and the percentage of haploid cells in tubular fragments from immature hamsters

The testes from immature hamsters undergo a spurt of growth, and it can be anticipated that the cellular composition of the testes used for the present experiments will show some variation. If the different preparations of tubular fragments contain different numbers of spermatids, different LDH-C₄ activities will be found. To validate the comparison of the results within a series of experiments using different cell preparations, the correlation between the percentage of haploid cells and the LDH-C₄ activity was estimated.

The distribution of cells according to their DNA contents is dependent on the age of the animal. Tubular fragments isolated from 25–40-day-old hamsters were analysed using DNA flow cytometry (Fig. 2). A strong correlation ($r = 0.996$) was observed for the LDH-C₄ activity/mg protein and the percentage of haploid cells (Fig. 3).

Effect of racemic gossypol on LDH-C₄ activity in tubular fragments from hamsters

The LDH-C₄ activity of tubular fragments isolated from 25-day-old hamsters was estimated before and after culture for 48 h in the absence or presence of racemic (+/–)-gossypol acetic acid. The medium was supplemented with 1% fetal calf serum (FCS) to improve attachment of the tubular fragments to the plastic culture wells. The presence of extracellular proteins, however, affects the toxicity of gossypol. It has been reported that gossypol binds to albumin, which reduces the effective free concentration (Haspel *et al.*, 1984).

After 48 h of culture in the absence of gossypol, a 1.5-fold increase of the specific activity of LDH-C₄ of the tubular fragments was observed (ratio $t = 48 \text{ h}/t = 0 \text{ h} > 1$) (Fig. 4). Using

Fig. 1. Enzyme kinetics of hamster LDH-C₄ using different substrates. The initial velocities, expressed as mU/mg protein, are plotted as a function of the substrate concentration (left), and also in double reciprocal plots (right). Assays were performed using the same amount of enzyme for all substrates. The substrates assayed were: (a) α-ketobutyrate (2-oxo-butanoate), (b) α-ketoisovalerate (2-oxo-3-methyl-butanoate), (c) α-ketoisocaproate (2-oxo-4-methyl-pentanoate).

different preparations of tubular fragments, this increase of the specific LDH-C₄ activity was $42 \pm 15\%$ (Student's *t* test, $P < 0.005$, $n = 5$). Preliminary evaluation indicated that this may represent a developmental increase of the percentage of spermatids in the culture (not shown). When the tubular fragments were cultured in the presence of 1–4 μM-racemic gossypol acetic acid, there was a dose-dependent suppression of the rise in LDH-C₄ activity. At a concentration of 4 μM, the specific activity of LDH-C₄ after 48 h of incubation was lower than that at the start of the incubation (ratio $t = 48 \text{ h}/t = 0 \text{ h} < 1$) (Fig. 4). These effects were long-term effects that did not become apparent before 24 h of incubation, but mainly from 24–48 h of incubation (results not shown). The results in Fig. 4 furthermore indicate that gossypol does not inhibit LDH-C₄ in the enzyme assay. The LDH-C₄ activities at $t = 0 \text{ h}$ were not lowered in the presence of gossypol, but a small stimulatory effect was observed with increasing doses of gossypol (Fig. 4).

Effect of (+)- and (–)-gossypol on the LDH-C₄ activity

A concentration of 4 μM of the (+)- and (–)-gossypol enantiomers in the presence of 1% FCS was used for incubation of tubular fragments for 48 h. The LDH-C₄ activity (U/mg protein) was

Fig. 2. DNA-flow cytometric analysis of the cellular composition of tubular fragments (spermatogenic epithelium) from hamsters at (a) 25 days and (b) 40 days of age.

Fig. 3. Correlation between LDH-C₄ activities and the number of haploid cells in tubular fragments isolated from 25–40-day-old hamsters. For each point, tubular fragments were prepared from 4 animals of the same age. The resulting line was evaluated by linear regression analysis ($r = 0.996$).

not affected by (+)gossypol, but (–)gossypol caused a marked inhibition of the LDH-C₄ activity as compared to the control incubations (Table 1). The variation of the LDH-C₄ activities in the different experiments (Table 1) is explained by the rapid testicular development in immature hamsters, as described above. Therefore, the results are also expressed as percentage of the control values (mean $\pm$ s.d.; $n = 6$) (Table 1).

Discussion

The lactate dehydrogenase isoenzyme LDH-C₄ is synthesized mainly in late-pachytene spermatocytes and in round spermatids (Hintz & Goldberg, 1977; Meistrich *et al.*, 1977), and development of the spermatogenic epithelium in immature animals leads to a rapid increase of the specific and total enzyme activity in the testis (Goldberg & Hawtrey, 1967). In the present

Fig. 4. Effect of racemic (+/-)-gossypol acetic acid on the LDH-C₄ activity of tubular fragments from 25-day-old hamsters, estimated at the start of the incubation (□—□) and after 48 h of incubation (○—○) in the presence of gossypol in medium containing 1% FCS. Each point represents tubular fragments from 4 culture wells. The ratio of the LDH-C₄ activities after and before incubation (ratio 48 h/0 h) is also presented (●—●).

Table 1. Effect of (+)- and (-)-gossypol on the LDH-C₄ activity of tubular fragments from hamsters, estimated after 48 h of incubation in the presence of gossypol in medium containing 1% FCS

Gossypol conc. (μM)	LDH-C ₄ activity (mU/mg protein)						LDH-C ₄ activity (% of controls) (mean ± s.d.)
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	
0 (control)	10.9	25.0	14.7	10.2	11.8	9.8	100
4.0 (+)	13.2	20.8	13.1	13.3	10.4	10.6	103.5 ± 19.6
4.0 (-)	6.2	14.0	8.6	7.9	6.4	5.5	59.5 ± 8.8*

*Significantly different from control values (Student's *t* test, *P* < 0.01).

experiments, a very good correlation between the percentage of haploid cells and the LDH-C₄ activity was observed for isolated tubular fragments from immature hamsters, at the stage of development when round spermatids appear in the testis. This correlation indicates that the LDH-C₄ activity present in the tubular fragments is almost exclusively derived from the haploid cells and not from spermatocytes, also because the correlation line, calculated by linear regression, intercepts the *y*-axis close to 0. It was shown that the isolated tubular fragments contained many spermatocytes, cells with a 4C amount of DNA. It would appear that these spermatocytes contain very little LDH-C₄ activity as compared to the spermatids. This point is under further investigation.

In the present in-vitro system of tubular fragments, gossypol caused a decrease of the LDH-C₄ activity (U/mg protein) as compared to control incubations. This has also been reported for in-vivo studies, in which a decrease of the specific activity of testicular LDH-C₄ was observed in gossypol-treated rats (Olgiati *et al.*, 1984a). An inhibitory effect of gossypol on cellular LDH-C₄ activity can be explained in two ways. A decrease of the enzyme activity can be caused by a direct effect of gossypol on the enzyme, but can also result from degeneration of spermatogenic cells and the subsequent loss of LDH-C₄ activity from the cells. Using DNA flow cytometry, this problem could not be solved effectively because non-viable cells may show an intact DNA content.

Direct inhibition of the LDH-C₄ enzyme by gossypol has been described by a number of authors. These observations were made in experiments using purified or partly purified

preparations of LDH-C₄ (Tso & Lee, 1982b; Morris *et al.*, 1986). However, gossypol has also been reported to inhibit other enzymes, such as adenylate cyclase (Olgati *et al.*, 1984b) and several enzymes of the citric acid cycle (Tso *et al.*, 1982). Furthermore, in experiments on direct inhibition of LDH-C₄ no specific effect of (–)gossypol was observed. Racemic gossypol and the (+)- and (–)-enantiomers of gossypol all showed a direct inhibitory effect on the activity of LDH-C₄ (Kim *et al.*, 1985; Whaley *et al.*, 1986). This may indicate that the specific effect of (–)gossypol on LDH-C₄ activity in the present in-vitro system involves a different mechanism of gossypol action.

However, we have observed that LDH-C₄ activity in isolated hamster spermatids was not inhibited by (–)gossypol, indicating that there is no direct effect on this enzyme (Den Boer & Grootegeod, 1988).

The (–)-enantiomer of gossypol but not the (+)-enantiomer inhibits proliferation of normal fibroblasts and tumour-derived cells (Joseph *et al.*, 1986). This indicates that the toxicity of (–)gossypol is not cell specific. However, it is possible that spermatids are much more sensitive to (–)gossypol than are other cell types.

In in-vitro experiments, the effects of gossypol on TM4 cells, a Sertoli cell line originally derived from immature mice, have been studied (Reyes *et al.*, 1986). Exposure of the TM4 cells to gossypol resulted in uncoupling of oxidative phosphorylation. In agreement with this, treatment of TR-ST cells, from a rat Sertoli cell tumour, with gossypol caused perturbation of mitochondrial rhodamine-123 accumulation (Tanphaichitr *et al.*, 1984). Rhodamine-123 accumulation in mitochondria is driven by the proton-motive force across the inner mitochondrial membrane, and H⁺-conducting uncouplers can therefore inhibit the rhodamine staining of mitochondria. Tanphaichitr *et al.* (1984) also observed that rhodamine-123 accumulation in mitochondria of isolated round spermatids was not sensitive to gossypol action. However, Den Boer & Grootegeod (1988) have reported marked inhibitory effects of low concentrations of gossypol on the ATP content of isolated round spermatids.

The presence of extracellular proteins greatly influences the cytotoxicity of gossypol. It has been reported that the cytotoxic effects of gossypol on cultured murine erythroleukaemia cells and embryonic lung cells can be partly or completely prevented by addition of fetal calf serum (Haspel *et al.*, 1984; Joseph *et al.*, 1986). Furthermore, the effect of gossypol is dependent on the total amount of cellular protein per incubation. A direct comparison of effects of gossypol on Sertoli cells and spermatids can be made if the different cell types are incubated under the same conditions, but this has not yet been done. The amount of gossypol that impaired the function of mitochondria of TM4 cells (in the absence of extracellular proteins) was 10 nmol per 2×10^5 cells (Robinson *et al.*, 1986). In contrast, 0.5 nmol gossypol per 2×10^5 spermatids (also in the absence of extracellular proteins) caused a rapid decrease of the ATP content of round spermatids (Den Boer & Grootegeod, 1988). From this, it may be concluded that spermatids are much more sensitive to gossypol action than are Sertoli cells. However, this point should be studied further using the different cell types from the same animal species, under identical incubation conditions.

The present study involves an in-vitro system of tubular fragments, containing Sertoli cells and spermatogenic cells. Using such a system, no conclusive information on the primary target cells of various compounds can be obtained. On the other hand, the tubular fragments may include possible Sertoli cell–germ cell interactions with respect to metabolic activation or inactivation of toxic compounds. Biochemical analysis of isolated tubular fragments could be useful to study the toxicity of gossypol derivatives, investigational antifertility agents other than gossypol, and various toxic agents.

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