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Comparison of sensitivities of rat spermatozoa, Sertoli and Leydig cells to gossypol acetic acid *in vitro* by LD_{50}

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ABSTRACT Rat epididymal spermatozoa, Sertoli and Leydig cells in culture were used to examine the direct effects of gossypol. The numbers of live spermatozoa were decreased drastically following 8 h incubation with increasing concentrations of gossypol up to 20 μg/ml, whereas the other cell types, especially the Leydig cells, were slightly affected in the same condition. By comparing the LD_{50}, the epididymal spermatozoa were most sensitive while the

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Leydig cells the least. There were no differences in responsiveness of the 3 types of cells to gossypol 40 μg/ml.

KEY WORDS gossypol acetic acid; spermatozoa; Sertoli cells; Leydig cells; cultured cells

The antifertility effects of gossypol on a variety of animals and man, aroused a great interest in assessing the intrinsic toxicogenic property of gossypol to monogastric animals. In rats, gossypol po. at low dose levels caused a degeneration of the mitochondrial sheath, cell membrane, and microtubules[1-3] in spermatozoa, but not Sertoli and Leydig cells. The tight junction of Sertoli cells changed only when the dosage of gossypol was doubled[4]. Further increases in dosages of gossypol caused functional changes of the Leydig cells[5-6]. Rat Sertoli cells were sensitive to gossypol *in vitro*[8], but the responses of spermatozoa and Leydig cells to gossypol have not been compared.

The purpose of this investigation was to examine the relative sensitivities of rat epididymal spermatozoa, Sertoli and Leydig cells to gossypol *in vitro*, as assessed by LD_{50}.

MATERIALS AND METHODS

Reagents and rats Sprague-Dawley rats were obtained from the Charles River Breeding Laboratories. Adult rats at the age of 60-70 d were used for collecting epididymal spermatozoa. Immature rats at the age of 13-19 d were used for Leydig cell and Sertoli cell preparations.

Media and sera were purchased from Gibco, insulin and transferrin from Sigma, Epidermal growth factor from Collaborative Research Inc, and Gossypol acetic acid (98% pure) from Shanghai Institute of Materia Medica.

Gossypol acetic acid powder was dissolved immediately before use in absolute alcohol as a stock solution. The gossypol acetic acid ethanol solution was added to the culture medium to yield a final concentration of 10-40 μg/ml. The ethanol content in the medium was no more than 0.1%.

Testicular cell culture Testicular cells were obtained and cultured as described by Zhuang *et al*[7].

1. Leydig cell-enriched preparation. The testes of 13-19 d old rats were decapsulated and incubated in 0.03% collagenase /dispase containing 0.003% soybean trypsin inhibitor (STI) to disperse the interstitial cells.

2. Sertoli cell-enriched preparation. The tubules were dispersed again into small clumps with collagenase. These clumps were washed by unit gravity sedimentation to remove single myoid and germ cells.

3. Culture medium. Both Leydig-enriched and Sertoli-enriched preparations were washed with the medium and plated into 35 × 10 mm Corning tissue culture dish. An inoculum of $4-8 \times 10^5$ Leydig cells and $1-2 \times 10^6$ Sertoli cells were plated and incubated in a 1:1 mixture of Ham's Nutrient F 12 and Dulbecco's modified Eagle' medium supplemented with insulin (Ins 10 μg/ml), transferrin (TF 5 μg/ml), and epidermal growth factor (EGF 10 ng/ml for 24 h to inhibit the growth of fibroblast cells.

After the 24 h incubation, the medium was changed to F 12/DME containing 5% horse serum and 2.5% new-born bovine serum. For Leydig cell culture human chorionic gonadotrophin (HCG) 25 ng/ml was added to the medium.

4. Count of Testicular cells. Cells were counted with a Coulter counter by trypan blue exclusion technique. Each culture had 3-5 replicate plates and the experiments were repeated 3 times.

Spermatozoa incubation Caudal epididymis isolated from adult rats were cut by scissors in pieces and incubated in the same

medium as the testicular cell culture at 37℃ for 10 min.

The spermatozoa in the suspension were transferred into culture dishes at a concentration of 10^6 cells/ml. Each dish contained gossypol acetic acid 10–40 μg/ml and ethanol no more than 0.1%.

Control culture dishes proved that, in the medium with 0.1% ethanol there was no deleterious effect on the viability of spermatozoa, Sertoli and Leydig cells during the 8 or 16 h incubation period. The spermatozoa were stained with eosin and nigrosin and examined for the mortality rate.

Data treatment LD_{50} and singnificant tests were calculated by probit analysis[8].

RESULTS

In the control medium, approximately 60% of spermatozoa and 100% of Sertoli and Leydig cells were alive at the end of 8 h incubation, regardless of the presence and absence of 0.1% ethanol. The viability of spermatozoa, Sertoli and Leydig cells exposed to gossypol acetic acid 10–40 μg/ml are shown in Fig 1. The number of viable cells in the control medium was considered as 100%. The numbers of live spermatozoa decreased drastically with increasing concentration of gossypol acetic acid up to 20 μg/ml. However, the viability of the two testicular cell types, especially Leydig cells, was slightly affected in a concentration up to 20 μg/ml of gossypol acetic acid. At this concentration, the live spermatozoa, Sertoli cells, and Leydig cells were 20±15%, 80±2% and 95±4% respectively after incubation for 8 h. The LD_{50} of gossypol acetic acid on spermatozoa, Sertoli and Leydig cells after 8 h exposure *in vitro* were 19.5(18.0–21.1), 25.4 (24.3–26.6) and 30.3 (29.3–31.4) μg/ml respectively (Tab 1). The epididymal spermatozoa were most sensitive and the Laydig cells the least.

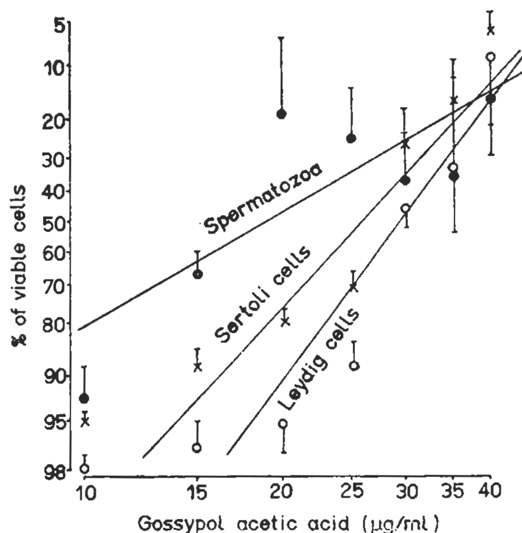

Fig 1. **Viable Sertoli cells (●), Leydig cells (○) and spermatozoa(×) exposed to gossypol acetic acid.**

Tab 1. **LD_{50} (μg/ml, 95% fiducial limits) of gossypol acetic acid to the cells of 3 types**

	LD_{50}	p value	
Sertoli cells	25.4(24.3–26.6)	<0.05 (Sertoli vs Leydig)	
Leydig cells	30.3(29.3–31.4)	<0.05 (Leydig vs Spermatozoa)	<0.05 (Sertoli vs Spermatozoa)
Spermatozoa	19.5(18.0–21.1)		

It was difficult to maintain the epididymal spermatozoa alive after 16 h incubation *in vitro* but not the Sertoli and Leydig cells. When the incubation period was extended to 16 h there was no significant difference in the sensitivity between the Sertoli cells and Leydig cells exposed to gossypol acetic acid (LD_{50}:24.5 μg/ml vs. 24.4 μg/ml).

DISCUSSION

The data presented here demonstrated that the rat epididymal spermatozoa were the most sensitive cells whereas the Leydig cells relatively insensitive to gossypol. However, in this experiment, the spermatozoa cells were obtained from the adult rats,

but other 2 types of testicular cells were from immature rats due to the methodological problem. A question should be raised whether the variety of sensitivity was caused by the difference in the age of animals. To explain this question, we have done an experiment in which the adult and immature rats showed no significant difference in the response to the antifertility effect of gossypol (Chang CC and Gu ZP, unpublished data).

The affinity of mitochondria of gossypol-treated cell to the labeled gossypol was about 2–3 times higher than that occurred in other organelles[9]. Gossypol inhibited the activities of pyruvate dehydrogenase complex and Na^+, K^+ and Mg^{2+}-dependent ATPases of sea urchin spermatozoa[10]. In addition, glucose and fructose metabolism of human spermatozoa were inhibited when incubated in the presence of gossypol. These findings indicate that gossypol acts on energy-related enzymes. This may be the cause that spermatozoa are more sensitive than Sertoli and Leydig cells to gossypol.

The results of the present experiment are in correspondence with the study of Zhuang *et al.*[7] showing that in a 6-d culture of rat testicular cells, the Sertoli cells is more sensitive than the Leydig cells to gossypol as evidenced by the conspicuous ultrastructural changes in the Sertoli cells but not in the Leydig cells.

We have previously demonstrated that there were no significant changes in testosterone or LH levels in rats receiving gossypol 7.5 and 15 mg/kg/d whereas the rats attained infertility[10]. Ultrastructural observations showed no detectable changes in the Leydig cells[2]. Administration of gossypol to rats at a high dose (30 mg/kg/d) caused significant reduction in testosterone. The results of the present *in vitro* experiment explains the *in vivo* finding[10].

REFERENCES

1 Dai RX, Pan SN, Liu ZI. Studies on the antifertility effect of gossypol II. A morphological analysis of the antifertility effect of gossypol. *Acta Biol Exp Sin* 1978; 11 : 27

2 Hoffer AP. Ultrastructural studies of the seminiferous and epithelium and epididymal sperm in rats treated with gossypol. *Arch Androl* 1982; 9 : 35

3 Nadakavukaren MJ, Sorensen RH, Tone NJ. Effect of gossypol on the ultrastructure of rat spermatozoa cell. *Tissues Res* 1979; 204 : 293

4 Gu ZP, Zong SD, Chang CC, Morphological changes in testes and epididymides of rats after gossypol. *Acta Pharmacol Sin* 1983; 4 : 40

5 Liang SX, Peng SN, Dong RH, Dai RX. Radio-immunoassay of serum T and LH in male rats administered gossypol. *Acta Biol Exp Sin* 1981; 14 : 191

6 Lin T, Murono FP, Osterman J, Nankin HR, Ciulson PB. Gossypol inhibits testicular steroidogenesis. *Fertil Steril* 1981; 35 : 564

7 Zhuang LZ, Phillips DM, Gunsalus GL, Bardin CW, Mather JP. Effects of gossypol on rat Sertoli and Leydig cells in primary culture and established cell lines. *J Androl* 1983; 4 : 336

8 Tanphaichitr N, Bellve Ar, Lampidis TJ, Chen LB. A direct effect of gossypol on rat Sertoli cells. *Biol Reprod* 1983; 28 (suppl 1) : 162

9 Xue SP, Liang DC, Fei RR, *et al.* Subcellular site of antispermatogenic effect of gossypol and its possible molecular mechanism of action. *Sci Sin* (series B) 1983; 26 : 614

10 Adeyemo O, Chang CY, Segal SJ, Koide SS. Gossypol action on the production and utilization of ATP in sea urchin spermatozoa. *Arch Androl* 1982; 9 : 343

11 Chang CC, Gu ZP, Tsong YY. Studies on gossypol I. toxicity, antifertility and endocrine analysis in male rats. *Int J Fertil* 1982; 27 : 213

以 LD_{50} 为指标比较大鼠附睾精子，睾丸支持和间质细胞对棉酚乙酸作用的敏感性

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提要 体外培养下，不同浓度的棉酚乙酸分别与大鼠附睾精子，睾丸支持细胞和间质细胞孵育 8 h，结果发现棉酚对三种细胞均有直接杀伤作用，其半数致死量 (LD_{50})分别为 19.5 (18.0－21.1)，25.4(24.3－26.6) 和 30.3 (29.3-31.4) μg/ml，统计学检验三者有显著差别。说明附睾精子对棉酚最为敏感，支持细胞次之，而间质细胞则相对不敏感。

关键词 棉酚乙酸； 精子； 塞尔托利细胞； 莱迪希细胞； 培养的细胞

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