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

ZHANG Lin-zhi (Institute of Zoology, Chinese Academy of Sciences, Beijing 100029, China)
GU Zhi-ping (Shanghai Int. of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China)
CHANG Chin-chuan (The Population Council, Center for Biomedical Research, New York NY 10011, USA)

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 48 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
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 antiestrogenic 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 mitochondrion shat, cell membrane, and microtubules in spermatozoa, but not Sertoli and Leydig cells. The tight junction of Sertoli cells changed only when the dosage of gossypol was doubled. Further increases in dosage of gossypol caused functional changes of the Leydig cells (1, 3-6). Rat Sertoli cells were sensitive to gossypol in vitro, 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 LD₅₀.

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. (10).

1. Leydig cell-enriched preparation. The testes of 13-19-d old rats were decapitated and incubated in 0.05% collagenase /dipase containing 0.065% 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 x 10 mm Corning tissue culture dish. An inoculun of 4-8 x 10⁵ Leydig cells and 1-2 x 10⁶ 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 (1 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 F12/DME containing 5% horse serum and 2.5% new-born bovine serum. For Leydig cell culture human chorionic gonadotropin (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 Casool epididymis isolated from adult rats were cut by scissors in pieces and incubated in the same
medium as the testicular cell culture at 37°C for 10 min.

The spermatozoa in the suspension were transferred to culture dishes at a concentration of 10^6 cells/ml. Each dish contained gossypol acetic acid 10-40 ug/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 LD₅₀ and significant tests were calculated by probit analysis³⁸. 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 ug/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 ug/ml. However, the viability of the two testicular cell types, especially Leydig cells, was slightly affected in a concentration up to 20 ug/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 LD₅₀ 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 38.3 (29.3-41.4) ug/ml respectively (Tab 1). The epididymal spermatozoa were most sensitive and the Leydig cells the least.

\[
\text{LD}_{50} \quad (\mu g/ml) \quad \text{95% Confidence limits} \\
\begin{array}{ll}
\text{Sertoli cells} & 25.4 (24.3-26.6) \\
\text{Leydig cells} & 38.3 (29.3-41.4) \\
\text{Spermatozoa} & 19.5 (18.0-21.1)
\end{array}
\]

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 (LD₅₀: 24.5 ug/ml vs. 24.4 ug/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 types of testicular cells were from immature rats due to the methodological problem. A question should be raised that 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 anti-antibody effect of gossypol (Chang CC and Gu ZP, unpublished data).

The affinity of mitochondria of gossypol-tREATED cell to the labeled gossypol was about 2-5 times higher than that occurred in other organs of rats (43). Gossypol inhibited the activities of pyruvate dehydrogenase complex and Na+, K+ and Mg2+-dependent ATPases of testis and epididymis spermatozoa (43). 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 studies of Zhuang et al. (43) showing that in a g-d culture of rat testicular cells, the Sertoli cells are 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 (43). Ultrastructural observations showed no detectable changes in the Leydig cells (41). Administration of gossypol to rats at a high dose (50 mg/kg/d) caused significant reduction in testosterone.

The results of the present in vitro experiment explains the in vivo finding (43).

REFERENCES

以 LD₅₀ 为指标比较大鼠附睾精液、睾丸支持和间质细胞对 棉酚乙酸作用的敏感性

庄 君 之 (中国科学院北京动物研究所，北京 100023, 中国) 顾 留 涛 (中国科学院上海有机化学研究所，上海 200031, 中国) 张 金 钧 (人口委员会遗传学研究所，纽约 10021, 美国)

摘要 体外培养下，不同浓度的棉酚乙酸分别与大鼠附睾精液、睾丸支持和间质细胞作用 8 h，结果表明棉酚乙酸对三类细胞均有不良影响作用，其中附睾精液 (LD₅₀) 分别为 19.6 (18.6～21.1), 25.4 (24.2～26.4) 和 25.2 (25.0～25.4) μg/ml。统计学检验显示有显著差

关键词 棉酚乙酸；精子；睾丸支持细胞