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EFFECT OF CLOMIPHENE ON TESTICULAR PROTEIN SYNTHESIS *IN VITRO*

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Abstract—Various concentrations of clomiphene citrate were incubated with rat testis tissue in order to study protein biosynthesis. The drug was found to inhibit the incorporation of labeled lysine into testicular proteins. The ED_{50} *in vitro* was 2×10^{-5}M. When this median effective dose was incubated with slices of rat liver, kidney and spleen, no significant effect on protein labeling was found. Both the *cis*- and *trans*-isomers of the drug are equipotent in inhibiting testicular protein labeling *in vitro*. It was concluded that these results may offer a partial explanation for the antispermatogenic effect of the drug observed *in vivo*.

CLOMIPHENE citrate (Clomid), 1-[*p*-(diethylaminoethoxy)-phenyl]-1,2-diphenyl-2-chloroethylene, is a synthetic nonsteroid compound originally synthesized as a possible contraceptive agent. Clomiphene does in fact induce a definite antifertility effect in the female, the mechanism of which remains unresolved.[1–3] Paradoxically, several clinical studies have also shown it to be efficacious in the treatment of human female infertility via induction of ovulation.[4–6]

Clomiphene has also been studied in the human male. Chronic administration of the drug to male volunteers has been reported to either enhance or suppress spermatogenesis depending upon the dose employed.[7] Low doses of clomiphene apparently stimulate the germinal epithelium indirectly through release of pituitary gonadotropins. Higher doses appear to mask the indirect effect by directly damaging the germinal epithelium. A direct effect of clomiphene on testicular function has also been suggested on the basis of experiments performed on gerbils.[8] Intratesticular injection of small doses of clomiphene results in necrosis of the seminiferous epithelium and of the Leydig cells.

Studies dealing with the possible mechanism of the direct inhibitory effect of clomiphene on the testis have not previously been reported. The present investigation was therefore designed in order to initially study the effect of clomiphene on one aspect of testicular function, specifically, the incorporation of radioactive lysine into proteins of the rat testis *in vitro*.

EXPERIMENTAL

Materials

The animals used in these experiments were male Sprague–Dawley rats weighing 300–400 g obtained from the Simonson Co. Berkeley, Calif. Clomiphene citrate was

generously supplied by the Wm. S. Merrell Company. It was prepared by homogenizing in distilled water with a motor-driven Teflon pestle (clearance 0·1–0·15 mm). L-Lysine-U-^{14}C with a specific activity of 312 mc/m-mole was obtained from Schwartz Bio-Research.

Incubation of the tissues

The rats were sacrificed by decapitation, the testes were removed and decapsulated and 150–200 mg of teased tissue was placed in each flask. Liver, kidney and spleen were sliced with the aid of a Stadie–Riggs microtome. The main chamber of the Warburg flask contained 2·9 ml of Krebs–Ringer bicarbonate buffer at pH 7·0. The drug was added in a volume of 0·1 ml. The side-arm contained $1{\cdot}3 \times 10^6$ disintegrations per minute (dis./min) of L-lysine-U-^{14}C in a volume of 0·2 ml. The final concentration of radioactive lysine in the incubation flask was 5×10^{-7}M. The gas phase was 95% O_2–5% CO_2. An incubation temperature of 32° was employed.[9] At the end of a 1-hr incubation period, the reaction was terminated by the addition of 0·3 ml of 5 N perchloric acid. Proteins were isolated as described by Busch *et al.*[10]

Radioactive assay

The dried protein pellets (10–15 mg) were dissolved in 1·0 ml of NCS solubilizer (Amersham/Searle) by heating at 60° for 10 min. Ten ml toluene scintillation fluid containing 0·5% 2,5-diphenyloxazole (PPO) and 0·006% *p*-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) was added to each sample. Radioactivity was determined in a Beckman LS-200B liquid scintillation spectrometer. Each sample was counted to a 2 σ pre-set error of $\pm$0·5 per cent. Quenching was determined with a ^{14}C-toluene internal standard. Efficiency ranged from 60 to 70 per cent.

RESULTS

The time course of the incorporation of L-lysine-U-^{14}C into proteins of rat testis incubated at 32° is presented in Fig. 1. A progressive increase in protein labeling occurred from 0 to 60 min, with increased incorporation of the tracer still present up to 120 min. From these data, a 1-hr incubation period was chosen as a convenient time for studying the effect of clomiphene on testicular protein labeling.

The effect of a range of concentrations of clomiphene on testicular protein labeling is shown in Fig. 2. The per cent inhibition of testicular protein labeling *in vitro* is plotted as a log-dose response. Clomiphene at a concentration of 10^{-5}M, or less, produced no statistically significant reduction in the incorporation of the tracer into testicular protein ($P > 0{\cdot}05$). However, at a concentration of 2×10^{-5}M, or more, protein labeling was significantly inhibited. From these data it would appear that the ED_{50} *in vitro* for clomiphene in this system is approximately 2×10^{-5}M.

In order to determine if the response of the testicular protein-synthesizing system to the addition of clomiphene *in vitro* was specific, three other tissues of the rat were investigated. Slices of liver, kidney and spleen were incubated with the median effective dose. The results are shown in Table 1. In none of the three tissues did clomiphene produce a statistically significant change in protein labeling *in vitro*.

In view of the fact that the clomiphene molecule contains an ethylene double bond in which the carbons have substituents, *cis* and *trans* geometric isomers exist. It therefore seemed of interest to determine if some stereospecificity existed with regard

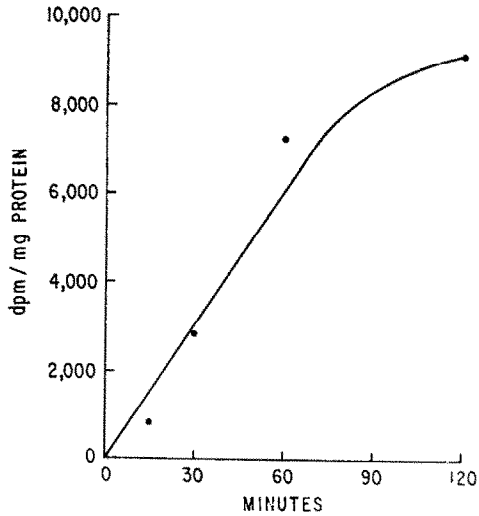

FIG. 1. Effect of incubation time on testicular protein labeling *in vitro*.

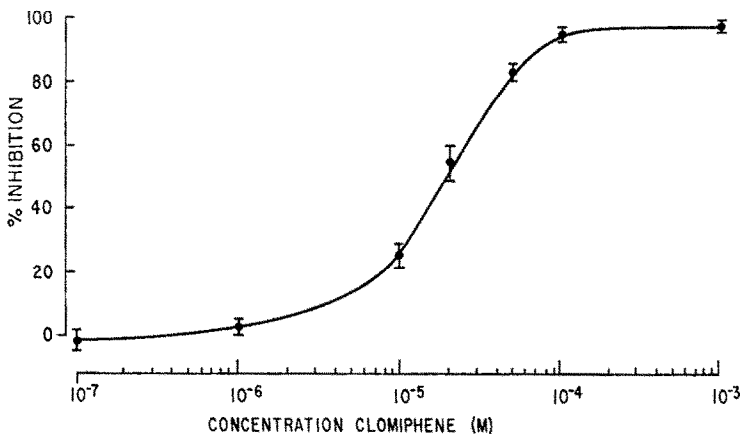

FIG. 2. Per cent inhibition of testicular protein labeling *in vitro* by varying concentrations of clomiphene. Control specific activity was 6870 ± 340 dis./min/mg of protein. Vertical bars represent standard error.

TABLE 1. EFFECT OF CLOMIPHENE CITRATE ON PROTEIN LABELING *IN VITRO* IN SLICES OF RAT LIVER, KIDNEY AND SPLEEN*

Tissue	Control (dis./min/mg protein)†	Clomiphene (dis./min/mg protein)
Liver	750 ± 40	610 ± 30 (NS)
Kidney	2680 ± 170	2350 ± 260 (NS)
Spleen	3000 ± 690	3090 ± 260 (NS)

* Tissue, 150–200 mg, incubated for 1 hr in Krebs–Ringer bicarbonate buffer, pH 7·0, in an atmosphere of 95% O_2–5% CO_2 at 32°. Final concentration of clomiphene was 2×10^{-5}M.

† Average of 3 experiments ± S. E.; NS = no statistically significant difference as compared to control value ($P > 0.05$).

TABLE 2. EFFECT OF *cis-* AND *trans*-CLOMIPHENE ON TESTICULAR PROTEIN LABELING *IN VITRO**

Drug†	Specific activity‡ (dis./min/mg protein)	Per cent inhibition
Control	10,130 ± 60	
cis-Clomiphene	4870 ± 370	52
trans-Clomiphene	4840 ± 240	52

* Testis, 150–200 mg, incubated for 1 hr in Krebs–Ringer bicarbonate buffer, pH 7·0, in an atmosphere of 95% O_2–5% CO_2 at 32°.
† Final concentration of *cis-* and *trans*-clomiphene in incubation flask, 2×10^{-5}M.
‡ Average of three experiments ± S. E.

to the inhibitory effect of clomiphene on testicular protein labeling *in vitro*. The results of these experiments are shown in Table 2. Incubation of testis tissue with either the *cis-* or *trans*-isomer of clomiphene at a concentration of 2×10^{-5}M resulted in an identical inhibitory effect.

DISCUSSION

The results of the present investigation indicate that clomiphene citrate is capable of blocking the incorporation of radioactive lysine into testicular proteins of the rat *in vitro*. That this effect of the drug may be specific for the testis is suggested by the fact that the protein-synthesizing system of neither rat liver, kidney nor spleen was altered by a median effective concentration.

The fact that both the *cis-* and *trans*-isomers of clomiphene are equipotent in inhibiting testicular protein synthesis suggests that interaction with the receptor in this system is not significantly influenced by the geometric position of the phenyl and Cl substituents on the ethylene carbon atoms. Studies *in vivo* dealing with biological specificity of the *cis-* and *trans*-isomers of clomiphene have yielded equivocal results. On the one hand, it has been shown that both *cis-* and *trans*-clomiphene possess estrogenic potency in restoring uterine weight in ovariectomized rats.[11] However, differences in the physiological activity of *cis-* and *trans*-clomiphene have been demonstrated with regard to their ability to modify the stimulation of uterine hexokinase activity by estradiol in chronic studies.[12]

The capacity of clomiphene citrate to impede directly spermatogenic function *in vivo* has been suggested from studies on both humans and rodents.[7,8] The fact that clomiphene is able to inhibit testicular protein biosynthesis *in vitro* may therefore offer a partial explanation for the degenerative changes observed in testes from animals receiving the drug. At present, neither the site of the inhibitory effect on protein synthesis nor which cell type of the spermatogenic cycle is specifically affected is known.

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