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Original article

Production of estradiol by the fetal rat testis

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Summary — Testes from 17- to 20-d-old rat fetuses were cultured *in vitro* for various time intervals in Medium 199 alone or with added gonadotrophins. Estradiol released into the culture media was determined by radioimmunoassay. A basal estradiol secretion rate was inexistent or undetectable at all stages studied. In early stages (17 and 18 d) there was no difference in the stimulatory effect of FSH or LH at the same concentration. At 19 d, the prevalence of FSH became apparent. At 20 d, a significant action of FSH was noted after only 3 h culture time. 1-Methyl-1,4-androstadiene-3,17-dione, an aromatase inhibitor, markedly depressed FSH-stimulated estradiol secretion. In the 20-d old testis, stimulation of estradiol production by FSH was more rapid and reached a higher level than by $(Bu)_2$cAMP. It is suggested that the difference in the action of LH and FSH reflects the difference in the time of appearance of the corresponding receptors in the developing fetal testis.

estradiol / gonadotrophin / fetal rat testis

Résumé — Production d'œstradiol par le testicule fœtal de rat. *Les testicules de fœtus de rat de 17 à 20 j, découpés en 2 ou 4 fragments suivant leur taille, ont été cultivés* in vitro *pendant des durées variables sur milieu 199 additionné ou non de gonadotrophines. L'œstradiol-17β libéré dans le milieu de culture a été dosé par radio-immunologie. Une sécrétion de base était inexistante ou non décelable à tous les stades. Aux stades de 17 et 18 j, pour une même concentration de LH ou de FSH, l'action stimulante des 2 gonadotrophines était la même. Au stade de 19 j, l'action de la FSH supplantait celle de la LH. Au stade de 20 j, l'action de la FSH était déjà décelable après 3 h de culture. L'inhibiteur d'aromatisation 1-méthyl-1,4-androstadiène-3,17-dione inhibait fortement la sécrétion d'œstradiol stimulée par la FSH. Au stade de 20 j, la stimulation de la production d'œstradiol était plus rapide et plus importante par la FSH que par le $(Bu)_2$AMPc. Il se peut que la différence d'action de la LH et de la FSH reflète la différence chronologique dans l'apparition des 2 sortes de récepteurs.*

estradiol / gonadrotrophine / testicule fœtal de rat

INTRODUCTION

It has been known for a long time that the horse testis secretes large amounts of estrone and estradiol (Zondek, 1934; Beall, 1940). Later studies showed that estrogen secretion is a general feature of the testis of adult mammals (man: Goldzieher and Roberts, 1952; Kelch *et al,* 1972; Longcope *et al,* 1972; Scholler *et al,* 1973; Baird *et al,* 1973; Weinstein *et al,* 1974; monkey : Kelch *et al,* 1972; dog : Kelch *et al,* 1972; rat : de Jong *et al,* 1973, 1974).

In fetal mammals, estrogen secretion by the testis has been first demonstrated by Attal (1969), who detected estrone and estradiol in testicular tissue of fetal sheep and measured their concentration therein. Human fetal testes can produce some estradiol *in vitro,* and this estradiol secretion increases slightly in the presence of hCG (Tapanainen *et al,* 1989). Aromatase activity has been demonstrated in the fetal rat testis by the conversion of [^{3}H]testosterone or [^{3}H]19-hydroxyandrostenedione into estradiol (Weniger and Zeis, 1988, 1990). In the present study, the endogenous production of estradiol by the fetal rat testis *in vitro* has been investigated.

MATERIAL AND METHODS

Rats of the Wistar strain have been used. The day following the night of cohabitation was considered as d 0 of gestation.

Organ culture

The testes were removed aseptically from 17–20-d-old fetuses. According to their size, they were each cut longitudinally into 2–4 thin pieces, which were cultured *in vitro* in 0.1 ml Medium 199 in a Nunc plastic Petri dish. The Petri dishes were placed in an airtight jar gassed with 95% O_2–5%CO_2 and incubated at 37°C for various time intervals. After the culture time, the media were inspected for infection and the explants were rinsed with 0.5 ml phosphate-buffered saline or 1 ml water. One explant in each experimental series was fixed for histological examination. The diluted culture media were kept at –20 °C until direct radioimmunoassay or extraction.

Radioimmunoassay

The assay buffer was gelatin-containing phosphate–buffered saline (pH 7.4) and the volume of the reaction mixture was 0.5 ml. 17β-[2,4,6,7-^{3}H] Estradiol (105 Ci/mmol) came from Du Pont de Nemours. The antiserum was a gift from Roussel–Uclaf (Romainville); it was directed toward 7-carboxymethyloxime estradiol-bovine serum albumin and was used at a final working dilution of 1/250 000. Standards ranged from 3–200 pg/tube. Free estradiol was adsorbed on a charcoal–dextran mixture. Samples were assayed in duplicate. All samples of a series were run in the same assay to avoid interassay variations.

Substances tested

Bovine LH (NIH-LH-B9) and ovine FSH (NIADDK-oFSH-17) gifts from the National Hormone and Pituitary Program, were used at concentrations of 0.01–10 μg/ml and $(Bu)_2$cAMP (Sigma) at a 0.5 mM concentration. 1-Methyl-1,4-androstadiene-3,17-dione, a gift from Schering (Berlin), was used in the range of 0.1–100 μM. Data shown are representative of at least 2 experiments of the same kind.

Statistical analysis

Results are given as the means ± SD, with *n* in parentheses. Differences between group means were analyzed by 1-way analysis of variance, followed by the Tukey test or Newman–Keuls test, if differences were significant. A P value of < 0.05 was considered significant. Regression analysis and Student's paired *t*-test were also employed (Zar, 1984).

RESULTS

Radioimmunoassay validation

In the direct radioimmunoassay performed on culture media of 20-d-old testes cultured for 24 h in the presence of LH (5 μg/ml), FSH (5 μg/ml) or $(Bu)_2$cAMP (0.5 mM) or in the absence of either of these substances, the percentage bound values were above the zero binding dose. After extraction with isooctane–ethyl acetate 7:3 (2 x 2 ml), the percentage bound values fell on the standard curve, and the amount of estradiol could be determined for each sample. So extraction was an obligatory step in the procedure.

The characteristics of the radioimmunoassay were the following. Total binding represented ≈ 50% of the radioactivity added. Non-specific binding was ≈ 3.5%. The sensitivity of the method, defined as the mass equivalent to twice the SD of zero binding was 5 pg. The accuracy of the method was evaluated by determining the recovery of known amounts of estradiol added to culture media before the extraction step or by the test of linearity. The recoveries of 12.5, 20 and 25 pg estradiol added to culture media were respectively 13.0 ± 1.4 (n = 4), 22.4 ± 3.6 (n = 12) and 26.0 ± 2.3 pg (n = 4). The differences between the theoretical and the determined values were evaluated by the paired-sample t-test and were found to be not significant. The linearity test performed on 10 samples at 4 different volumes (25, 50, 100 and 200 μl) showed that the amount of estradiol was proportional to the volume of extract (r = 0.9898). The intra- and interassay coefficients of variation were respectively 3.2 ± 3.4% (n = 11) and 10.5 ± 7.6% (n = 22). Net sample values were obtained after subtracting the extraction blank.

Influence of FSH and LH and culture time

Twenty-d-old testes were cultured for 3,6 or 24 h in the presence of LH (5 μg/ml), FSH (5 μg/ml) or $(Bu)_2$cAMP (0.5 mM) or in the absence of either of these substances. As seen in table I and figure 1, a basal estradiol secretion rate was inexistent or very low. The stimulatory effect of FSH was the most rapid, estradiol being measurable after only 3 h of culture. The stimulatory effect of LH was obvious after 6 h of culture and increased significantly between 6 and 24 h. In the presence of $(Bu)_2$cAMP the release of estradiol was proportional to culture time between 6 and 24 h. The action of FSH was most conspicuous between 3 and 6 h.

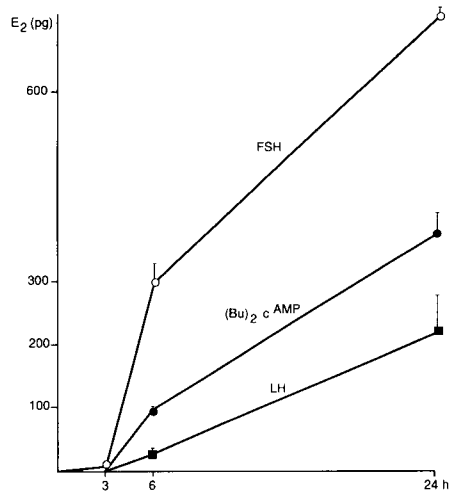

Fig 1. Time-course study of the effects of LH (5 μg/ml), FSH (5 μg/ml) or $(Bu)_2$cAMP (0.5 mM) on estradiol release by 20-d-old fetal rat testes cultured *in vitro* for 3, 6 or 24 h. Each point represents the mean of 3 determinations. Bars: SD.

Table I. Release of estradiol (in pg) by 20-d-old fetal rat testes cultured in the presence of LH (5 µg/ml), FSH (5 µg/ml) or $(Bu)_2$cAMP (0.5 mM) for 3, 6 or 24 h.

Culture period (h)	*Control*	*LH*	*FSH*	*$(Bu)_2$cAMP*
3	Undetected (3)	Undetected (3)	7.6 ± 2.0 (3)	Undetected (3)
6	Undetected (3)	26.5 ± 8.5 (3)	297 ± 29 (3)	97 ± 2.3 (3)
24	Undetected (3)	217 ± 59 (3)	723 ± 16 (3)	379 ± 36 (3)

Mean ± SE; *n* in parentheses.

Influence of the concentration of stimulating substances

As seen in figures 2 and 3, the production of estradiol responds in a dose-dependent manner to increasing concentrations of FSH or LH.

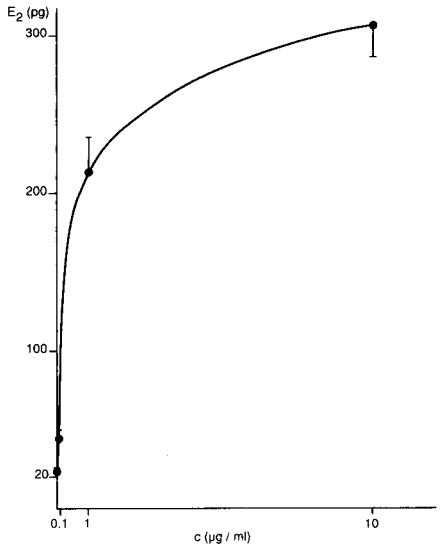

Fig 2. Dose–response curve for the production of estradiol by 20-d-old fetal rat testes cultured for 6 h in the presence of FSH (0.01–10 µg/ml). Each point represents the mean of 5 determinations. Bars: SD. The values are significantly different from one another.

Influence of the age of the testis

Estradiol secretion can be stimulated in the fetal rat testis by both FSH and LH as early as 17 d old. As seen in figure 4, LH seemed more effective than FSH at concentrations of 0.1 and 1 µg/ml at 17 and 18 d, although the difference was not significant. At a concentration of 10 µg/ml, the stimulatory effect of FSH exceeded that of LH, the difference being significant at 19 d.

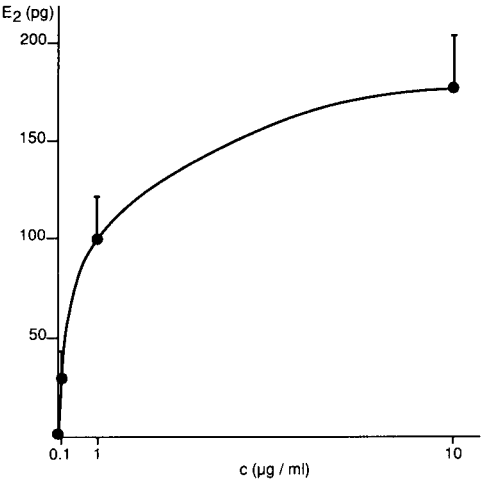

Fig 3. Dose–response curve for the production of estradiol by 19-d-old fetal rat testes cultured for 24 h in the presence of LH (0.01–10 µg/ml). Each point represents the mean of 3 determinations. Bars: SD. The values are significantly different from one another.

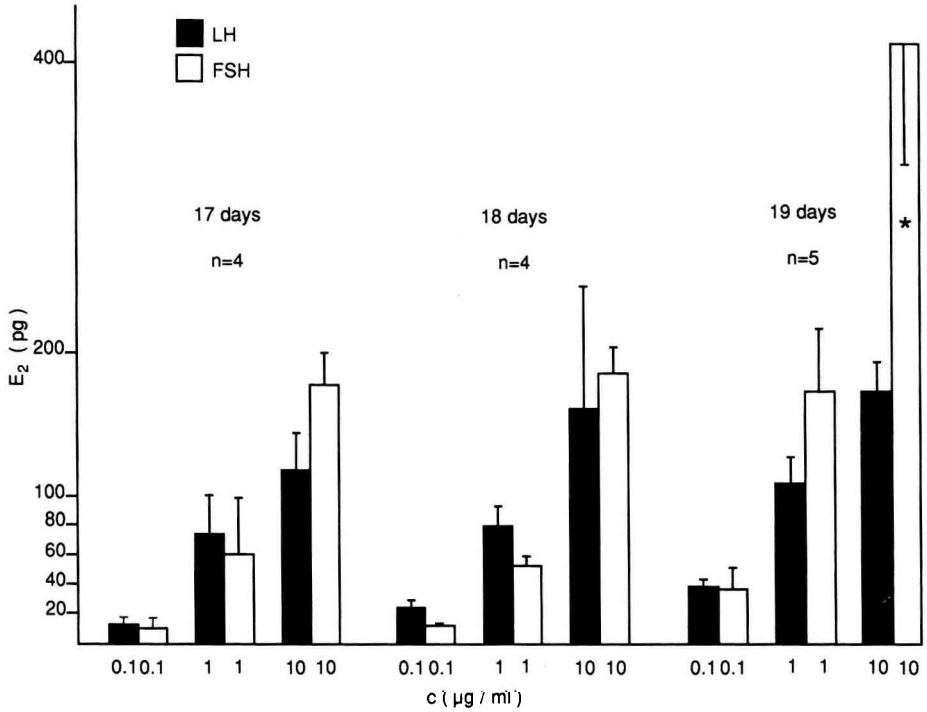

Fig 4. Diagrammatic representation of estradiol release by 17- to 19-d-old fetal rat testes in the presence of 0.1, 1 or 10 µg/ml of LH or FSH. Four determinations were made at 17 and 18 d, and 5 at 19 d. Bars represent the SD. At no stage did estradiol release differ significantly between LH and FSH at the same concentration of LH or FSH, except at the 19-d stage at a concentration of 10 µg/ml*. A basal estradiol secretion rate was inexistent or undetectable.

Influence of aromatase inhibitors

Inhibition of FSH-stimulated estradiol secretion by 1-methyl-1,4-androstadiene-3,17-dione is shown in figure 5.

DISCUSSION

In previous studies, the presence of aromatase activity in the fetal rat testis was shown by the conversion of tritiated testosterone or 19-hydroxyandrostenedione into estradiol (Weniger and Zeis, 1983, 1987, 1988, 1990). These results have been corroborated in the present investigation, which demonstrates *de novo* estradiol production under the stimulating influence of FSH or LH.

In other studies, hCG slightly stimulated the production of estradiol by minced 19- to 23-wk-old human fetal testes (Tapanainen *et al,* 1989). LH induced a 30% increase in aromatase activity of isolated Leydig cells from 21-d old fetal rat testes cultured *in vitro* for 3 d (Tsai-Morris *et al,* 1986). However, LH did not stimulate the aromatization of 19-hydroxyandrostenedione by the 20-d-old fetal rat testis (Weniger and Zeis, 1990).

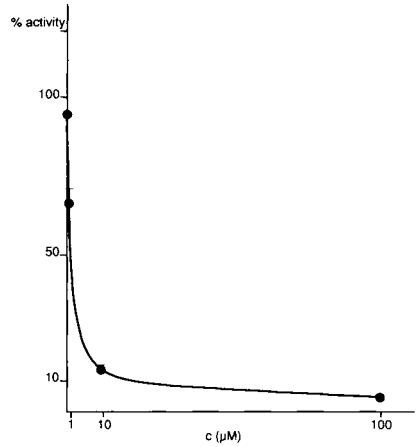

Fig 5. Inhibition of FSH-stimulated (5 μg/ml) aromatase of 20-d-old fetal rat testis by 1-methyl-1,4-androstadiene-3,17-dione (0.1–100 μM). Each point represents the mean of 4 determinations. Bars: SD. Inhibition at a 1-μM concentration was significant, but was not at a 0.1-μM concentration. Culture period: 6 h.

In the present investigation, both LH and FSH stimulated estradiol secretion by the fetal rat testis in the same range when used at the same concentration, at least at an early stage (17 and 18 d). At 19 d, the stimulatory effect of FSH exceeded that of LH, and this trend continued at 20 d. At this stage, the effect of FSH was noticeable after only 3 h of culture, whereas LH needed 6 h to elicit a measurable effect. This difference in LH and FSH action is most probably due to the difference in the time of appearance of the LH and FSH receptors. LH receptors were first detected in the fetal rat testis at 15.5 d of gestation, whereas FSH receptors could not be measured before 17.5 d of gestation (Warren *et al,* 1984). On the other hand, since LH did not stimulate the conversion of [^{3}H]19-hydroxyandrostenedione into estradiol, it probably favoured the *de novo* production of estradiol by increasing the availability of precursor testosterone, whereas FSH directly stimulated the aromatase enzyme system. In this way, one can understand that the action of LH drops behind that of FSH with increasing age, the secretion of testosterone already being maximally stimulated. On the contrary, FSH receptor levels rose sharply at 20.5 d, and the explanation of the intense action of FSH at this stage may be seen therein. As regards the fact that $(Bu)_2$cAMP was less active than FSH in stimulating estradiol secretion by 20-d-old fetal rat testis, it may be supposed that the FSH receptor mechanism is more effective in producing a high intracellular cAMP concentration than passive penetration of $(Bu)_2$cAMP into the cell.

Hypotheses have been put forward regarding the role of estrogen in testicular development. As in the testis of the adult rat (Dorrington and Armstrong, 1975; Valladares and Payne, 1979), in the fetal rat testis, estrogen produced by the Sertoli cells might exert a direct inhibitory effect on testosterone synthesis in Leydig cells. In cultures of isolated fetal rat Leydig cells, estradiol indeed inhibited testosterone synthesis (Tsai-Morris *et al,* 1986). Estrogen could also serve to increase the number of estrogen binding sites in fetal testis Leydig cells. Furthermore, estradiol could be involved in gene transcription of microsomal P-450 enzymes (Tsai-Morris *et al,* 1986).

To conclude, as the FSH stimulation of aromatase has been demonstrated *in vitro,* might this regulatory mechanism be operative *in vivo*? FSH cells have been revealed by immunocytochemistry in the adenohypophysis of rat fetuses from 19 d of gestation (Tougard *et al,* 1977; Watanabe and Daikoku, 1979), and FSH has been detected by radioimmunoassay in the pituitary of male fetuses from 17 d of gestation (Chowdhury and Steinberger, 1986). Increased production of estradiol under the influence of FSH could play a part in the

decline of testosterone secretion by the fetal testis before birth.

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