Follicles explanted from pentobarbitone-treated rats provide a model for atresia*

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Summary. Graafian follicles from rats treated for 1 or 2 days with pentobarbitone sodium (Nembutal) were similar in appearance to pro-oestrous preovulatory follicles, but after 3 or 4 days of treatment early atretic changes were recognized. Ovulatory efficiency decreased to 88, 70, 52 and 31% after 1, 2, 3 and 4 days of treatment, respectively. The mean $\pm$ s.e.m. rate of accumulation (ng/follicle/24 h) of progesterone, androstenedione and oestradiol was 3.6 ± 0.7 , 4.0 ± 0.3 and 18.9 ± 3.9 respectively in preovulatory follicles and 10.2 ± 1.7 , 0.9 ± 0.1 and 1.9 ± 0.4 respectively in follicles explanted from rats treated for 4 days with Nembutal. Addition of LH (5 μ g/ml) to the culture medium stimulated steroid accumulation by both types of follicles. Thus atretic follicles are characterized by impaired androgen and oestradiol formation. Addition of testosterone (1 μ g/ml) to the culture medium increased the accumulation of oestradiol by atretic follicles. It is inferred that the early stages of atresia of rat follicles are distinguished by a deficiency in the activity of enzymes responsible for the conversion of progesterone to androgens that can serve as substrates for aromatization.

Introduction

The vast majority of the oocyte and follicular populations undergoes atresia rather than ovulation (reviewed by Byskov, 1978). Nevertheless, the factors which determine whether a follicle will ovulate or undergo atresia are still obscure. This is probably because incipient atresia is recognized only in retrospect. Therefore the development of an animal model in which atresia can be produced in a synchronized follicle population offers an attractive approach to the study of various aspects of atresia.

The administration of pentobarbitone sodium on the afternoon of pro-oestrus blocks the preovulatory surge of gonadotrophins and ovulation is prevented (Everett & Sawyer, 1950; Naftolin, Brown-Grant & Corker, 1972; Ayalon, Tsafiri, Lindner, Cordova & Harell, 1972; Butcher, Collins & Fugo, 1974; Ashiru & Blake, 1978). Daily administration of the drug, starting on the day of pro-oestrus, results in atresia of Graafian follicles within 3 days (Everett & Sawyer, 1950). Atresia induced in this way may be suitable for study of the physiological changes within the follicle before the first morphological signs of atresia can be observed. However, in this model only atresia of mature Graafian follicles which have attained full responsiveness to LH can be studied.

The purpose of this study was to examine the relationship between follicular histology, the ability of the follicle to ovulate in response to hCG and the pattern of follicular steroidogenesis *in vitro* in animals treated with pentobarbitone sodium.

* Presented as the Arthur Walpole Memorial Lecture to the Society of the Study of Fertility, Glasgow, 1979.

Materials and Methods

Animals

The Wistar-derived rats were from the departmental colony and were housed in air-conditioned rooms with light between 05:00 and 19:00 h. Pelleted food and water were always available. The animals were 4 months old and daily vaginal smears were taken to confirm that each had shown at least two normal 4-day cycles immediately before the experiment.

Pentobarbitone sodium (Nembutal, Abbott; 30 mg/kg body weight) was injected intraperitoneally on the day of pro-oestrus between 13:40 and 14:00 h and on the next 3 days between 13:30–14:00 and 15:40–16:00 h. The critical period for blockage of ovulation by Nembutal in our colony is between 13:30 and 16:30 h (Tsafiriri & Kraicer, 1972). Immediately after the last injection of Nembutal, some of the rats (5–10/group) were given an i.p. injection of 4 i.u. hCG (Pregnyl, Organon). Ovulation was checked by expressing and counting tubal ova 16–18 h after hCG.

Culture of follicles

Animals were killed by cervical dislocation between 08:00 and 12:00 h on the day after the last Nembutal injection. Graafian follicles were explanted from the ovaries and cultured as previously described (Tsafiriri, Lindner, Zor & Lamprecht, 1972). Preovulatory follicles explanted between 08:00 and 12:00 on the day of pro-oestrus from untreated females were used as normal controls. Each dish contained 2–3 follicles, and ovine LH (NIH-LH-S18; 5 µg/ml), FSH (NIH-FSH-S11; 10 µg/ml) or testosterone (Ikapharm; 1 µg/ml) were added to the medium as indicated. The gonadotrophins were dissolved in 0.9% (w/v) NaCl and testosterone in absolute ethanol before dilution in the medium. The final ethanol concentration of 0.1% had no effect on steroid accumulation.

The medium was collected after 6 h, replaced by fresh medium, and collected again after further incubation for 18 h.

Steroid assays

The amounts of progesterone, androstenedione, testosterone and oestradiol-17β in the medium were determined by radioimmunoassays as described by Lindner & Bauminger (1974). Steroids were extracted from the medium with 8 volumes ether and the ether extracts were evaporated to dryness under N₂. The residue was redissolved in ethanol and portions were taken for steroid determination. Well characterized and specific antisera against progesterone, androstenedione, testosterone and oestradiol-17β were obtained by immunizing rabbits with albumin conjugates of the following steroid derivatives: progesterone-11α-hemisuccinate, the 7-carboxyethylthioethers of androstenedione and testosterone and the 6-carboxymethyl oxime of oestradiol (Bauminger, Kohen & Lindner, 1974; Kohen, Bauminger & Lindner, 1975). The major cross-reactions of the anti-progesterone and anti-androgen sera were with the corresponding 5α-pregnane and 5α-androstane compounds and the anti-oestradiol sera cross-reacted markedly with 6-oxo- and 6-hydroxy-oestradiol (Bauminger *et al.*, 1974). The sensitivities and intra- and inter-assay coefficients of variation for the progesterone, androstenedione, testosterone and oestradiol assays were 15, 30, 30 and 20 pg/ml and 2, 3, 4 and 3%, and 20, 16, 18 and 20% respectively.

The statistical differences between groups were calculated by Student's *t* test.

Histological procedures

Some of the ovaries (3–4/group) were used for morphological examination. They were fixed in Bouin's solution, dehydrated, embedded in paraffin wax and cut serially at 7 μ m. Sections were stained with haematoxylin and eosin and all the large Graafian follicles were examined for signs of atresia.

Results

Morphology of the Graafian follicles after Nembutal treatment

Follicles from rats after 1 or 2 days of Nembutal treatment were similar in appearance to the preovulatory follicles. They contained an oocyte in the dictyate stage, a few dividing granulosa cells, and no pyknotic granulosa cell nuclei.

After 3 and 4 days, most of the follicles contained an oocyte in the dictyate stage. However, in some of the oocytes meiosis-like changes, such as chromosomes at metaphase or the presence of a polar body, could be observed after 4 days of Nembutal treatment. Pyknotic nuclei were present in the granulosa layer but mitotic figures could also still be seen. The granulosa layer and basement membrane appeared puckered. Hence, according to our classification (Braw & Tsafiriri, 1980) most of the large follicles had reached Stage I of atresia by Day 3, and Stages I and II of atresia by 4 days after the start of Nembutal treatment.

Induction of ovulation by hCG

The percentage of rats ovulating following hCG administration was only slightly decreased following Nembutal treatment but the number of ova shed and the ovulatory efficiency was significantly affected (Table 1).

Table 1. Induction of ovulation by hCG in pentobarbitone-treated rats

	Days of pentobarbitone sodium treatment				
	0	1	2	3	4
No. of rats treated	12	10	10	5	10
No. of rats ovulating (%)	12 (100)	9 (90)	8 (80)	4 (80)	7 (70)
No. of ova shed/rat treated	11.0 $\pm$ 0.6	9.7 $\pm$ 1.1	7.7 $\pm$ 1.4*	5.8 $\pm$ 1.7***	3.4 $\pm$ 1.3***
Ovulatory efficiency (%)†	100	88	70*	53**	31***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with Day 0.

† (No. of ova/treated rat $\times$ 100)/(no. of ova/control rat).

Follicular steroidogenesis

The changes in steroidogenesis after 6 and 24 h of culture were similar and therefore only the 24 h data are illustrated and discussed.

Preovulatory follicles incubated in LH-free medium accumulated predominantly oestradiol-17 β , while production of progesterone, androstenedione and testosterone was low. In follicles from Nembutal-treated animals the production of oestradiol-17 β , androstenedione and testosterone was reduced, while progesterone accumulation increased (Text-fig. 1), being significantly higher on Day 1 ($P < 0.01$) and on Days 2, 3 and 4 ($P < 0.001$). The ratio of oestradiol-17 β /progesterone accumulation after 24 h of incubation was 5 for the preovulatory follicles, 1 for follicles after 1 day and 0.2 after 4 days of Nembutal treatment.

Text-fig. 1. Accumulation of progesterone (P), androstenedione (A), testosterone (T) and oestradiol-17β (E₂) by follicles from pentobarbitone sodium-treated rats during 24 h of culture in the hormone-free medium and in medium containing 5 μg LH/ml. Each bar represents the mean ± s.e.m. of 6–10 determinations. The data are expressed as ng steroid/follicle; the wet weight of a follicle is about 150 μg with 15–20 μg protein/follicle.

Addition of LH to the culture medium increased steroid production in all types of follicles. Progesterone accumulation was significantly higher ($P < 0.001$) after Nembutal treatment than in the preovulatory follicles. A 50% or more reduction in formation of oestradiol-17β, testosterone and androstenedione was observed in the follicles from rats that had received Nembutal.

Oestradiol-17β formation in the presence of testosterone

As shown in Table 2, testosterone (1 μg/ml) in the medium significantly increased production of oestradiol-17β in preovulatory follicles and in follicles from Nembutal-treated rats ($P < 0.05$). Addition of LH in the presence of testosterone increased the accumulation of oestradiol-17β in follicles from Nembutal-treated rats and restored oestradiol levels to those of untreated preovulatory follicles. Although FSH increased oestradiol-17β accumulation, values on Day 4 were not as high as those on the day of pro-oestrus.

Table 2. Accumulation during 6 h of culture of oestradiol-17 β (ng/follicle) in a medium without (–T) or with (+T) addition of testosterone (1 μ g/ml), by follicles from rats treated with pentobarbitone sodium for 0, 1 or 4 days

Other medium additions	Day 0		Day 1		Day 4	
	–T	+T	–T	+T	–T	+T
None	7.5 $\pm$ 0.2	15.9 $\pm$ 2.0*	3.1 $\pm$ 0.4	9.7 $\pm$ 0.8*	1.0 $\pm$ 0.2	9.8 $\pm$ 3.0*
LH	26.2 $\pm$ 3.0	20.8 $\pm$ 1.4	19.6 $\pm$ 1.7	32.6 $\pm$ 2.1*	4.7 $\pm$ 0.8	16.9 $\pm$ 2.8**
FSH	25.1 $\pm$ 2.5	21.7 $\pm$ 2.3	23.2 $\pm$ 1.1	44.5 $\pm$ 3.2*	7.4 $\pm$ 0.4	10.2 $\pm$ 2.4

Values represent mean $\pm$ s.e.m. of 3–10 cultures and 2–3 follicles/culture.

* $P < 0.01$; ** $P < 0.001$ compared with corresponding –T values.

Discussion

Graafian follicles from rats treated with Nembutal for 1 or 2 days showed no morphological signs of atresia. Most of these follicles were still able to ovulate as shown by their response to hCG administration. The first morphological sign of atresia (pyknotic granulosa cell nuclei) appeared in the follicles after treatment of the rats with Nembutal for 3 or 4 days and these follicles gave only a partial response to hCG. It therefore appears that 3–4 days of Nembutal treatment are needed for preovulatory follicles to become atretic. This confirms previous observations of Everett & Sawyer (1950). On the other hand, it has been shown that atretic changes occur in rat follicles 19 h after hypophysectomy and most of the follicles of such animals fail to respond to the hCG even 12 h after the operation (Talbert, Meyer & McShan, 1951). This discrepancy can be explained by the fact that, unlike hypophysectomy, pentobarbitone sodium blocks only the gonadotrophin surge while basal levels of serum gonadotrophins remain unaffected (Daane & Parlow, 1971; Ashiru & Blake, 1978). The presence of basal levels of gonadotrophins may delay atresia of preovulatory follicles and PMSG has in fact been shown to prevent atresia in mice and rats (Peters, Byskov, Himelstein-Braw & Faber, 1975; Peters, 1979; Braw & Tsafiriri, 1980).

The present study shows that the steroidogenic activity of atretic follicles is markedly different from that of preovulatory ones. Furthermore the results indicate that changes in steroid secretion occur very early during the atretic process, i.e. before morphological signs of atresia can be observed. Thus after 1 day of Nembutal treatment progesterone accumulation was doubled in gonadotrophin-free medium and increased 4–6-fold in response to the addition of LH (Text-fig. 1). Whether this increase in progesterone accumulation is related to atretic changes within the follicle or is due to another, yet undetermined, process remains to be established. An increase in progesterone accumulation has been demonstrated in atretic sheep follicles (Moor, Hay, Dott & Cran, 1978; Hay, Moor, Cran & Dott, 1979).

Follicular oestradiol synthesis decreased with the length of Nembutal treatment. Thus atretic follicles after 4 days of Nembutal treatment accumulated only one-fifth as much oestradiol as did preovulatory follicles. A similar decrease in oestradiol accumulation was observed by Moor *et al.* (1978) in cultures of sheep atretic follicles. Likewise the oestradiol content of mare atretic follicles was markedly reduced compared to that of healthy follicles (Condon, Ganjam, Kenney & Channing, 1979).

In cultures of rat atretic follicles androgen secretion was reduced although Moor *et al.* (1978) found a 2–3-fold increase for sheep follicles in culture. Moor *et al.* (1978) concluded that during atresia of sheep follicles aromatizing enzymes were affected. However, our results with rats show that addition of testosterone increased oestradiol production to levels similar to those of untreated preovulatory follicles (Table 2). In the rat, therefore, one or more of the enzymes

responsible for the synthesis of androgen from progesterone, rather than aromatizing enzymes, seem to be affected during early atretic changes. The discrepancy between our results and those of Moor *et al.* (1978) may be due to species differences and/or to the stage of atresia at which the follicles were explanted. It is possible that the rat follicles were explanted at an earlier stage of atresia.

Atretic rat follicles responded to LH with increased steroidogenesis which was due entirely to enhanced progesterone synthesis because androgen and oestradiol accumulation was only one-ninth that seen in preovulatory follicles. These results indicate that in early stages of Nembutal-induced follicular atresia in the rat, steroidogenic responsiveness to LH was not impaired.

Our preliminary study of atretic follicles following hypophysectomy indicate changes in steroidogenesis similar to those observed in the follicles following Nembutal treatment, i.e. an increase in progesterone and a decrease in androgen and oestradiol secretion. However, these changes appeared much earlier as compared to pentobarbitone-treated rats. Thus it appears that pentobarbitone treatment brings about changes analogous to these in atretic follicles obtained by total gonadotrophin deprivation, but the process of atresia is slowed down in the former. The occurrence of atresia in rats treated with pentobarbitone sodium will clearly be useful for further study of extragonadal and follicular factors involved in the atretic process.

We thank the NIAMDD for the LH and FSH preparations; Professor H. R. Lindner for his interest; Dr F. Kohen and Dr A. Nimrod for advice; Mrs A. Tsafiriri for skilful technical assistance; and Mr S. Yosef for care of the animals. The contribution of R.H.B. constitutes part of the requirements for the Ph.D. degree of the Feinberg Graduate School of the Weizmann Institute of Science. The work was generously supported by the Population Council, New York, and the Ford Foundation.

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Received 13 July 1979