Effect of RU 486 on luteal function in the early pregnant rat*

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Summary. A dose of 30 mg RU 486/kg, an antiprogesterone, was administered to pregnant rats on Day 2 (Group 1) or Day 4 (Group 2) of pregnancy. RU 486 significantly changed serum progesterone and oestradiol concentrations and luteal 3β -HSD and 20α -HSD activities in Group 1, and implantation was significantly inhibited. The luteal 3β -HSD activity in Group 2 rats on Day 6 was significantly ($P < 0.01$) lower than the control value (7.5 ± 0.6 and 10.1 ± 0.6 mU/mg protein respectively). This decline in the 3β -HSD activity was followed by a marked decrease in the serum progesterone concentration, resulting in a significant decrease of the progesterone/oestradiol ratio and implantation was completely inhibited. The 20α -HSD activity, which could not be detected on Day 6 in the control rats, was twice as great in Group 2 than in Group 1 rats (17.5 ± 1.2 and 7.4 ± 3.1 mU/mg protein respectively). Ultrastructural examination of corpora lutea of Group 2 rats confirmed luteolysis. These results suggest that RU 486 has a luteolytic effect and its anti-implantation effect is concomitant with luteolysis of the corpora lutea of pregnancy.

Keywords: RU 486; corpora lutea; luteolysis; 3β -HSD; 20α -HSD; rat

Introduction

RU 486 [17 β -hydroxy-11 β -(4-dimethylaminophenyl)-17 α -(1-propynyl) oestra-4,9-dien-3-one] is a synthetic steroid which binds to endometrial progesterone receptors and acts as a progesterone antagonist (Healy *et al.*, 1983; Gravanis *et al.*, 1985). It has been shown that RU 486 prevents implantation in rats, and Philibert *et al.* (1985) reported that implantation was inhibited when a single dose (30 or 100 mg/kg) of RU 486 was administered on any day between Days 2 and 6 after mating. However, the question of whether the anti-implantation effect of RU 486 is mediated by the changes in ovarian steroid hormones has not been studied.

Dimattina *et al.* (1986) observed that RU 486 induced a significant decrease in the activity of 3β -hydroxysteroid dehydrogenase (3β -HSD) concomitant with a decrease in progesterone secretion by human granulosa cell cultures. In addition, 20α -hydroxysteroid dehydrogenase (20α -HSD) is known to regulate the secretion of progesterone from corpora lutea (Kuhn & Briley, 1970), and a marked increase in activity has been observed in regressing corpora lutea (CL) of pregnant rats treated with abortifacient agents (Okazaki *et al.*, 1976).

To elucidate the effect of RU 486 on luteal function, a dose capable of inhibiting implantation was given to pregnant rats on Days 2 or 4 after mating, and the number of implantation sites, serum progesterone and oestradiol concentrations, and luteal 3β -HSD and 20α -HSD activities were examined. Morphological characteristics of CL were also investigated.

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Materials and Methods

Animals. Female Wistar strain rats, 10 weeks old, were kept in an air-conditioned room at 23°C with 12 h of artificial light and 12 h of darkness daily, with free access to pelleted food and water. They were paired with mature male rats, and vaginal smears were examined to confirm impregnation. Day 1 of pregnancy was the day when spermatozoa were found in the vaginal smears. In Exp. I, the animals were injected subcutaneously with 30 mg RU 486/kg body weight (Roussel-Uclaf, Romainville, France), dissolved in 70% ethanol (20 mg/ml) on Day 2 (Group 1) or Day 4 (Group 2), and killed on Day 6 to count the number of implantation sites. At autopsy, blood samples were obtained from the posterior vena cava to assay serum progesterone and oestradiol concentrations. CL were enucleated from the excised ovaries to measure 3 β -HSD and 20 α -HSD activities. Parts of the CL were processed for electron microscopy. Rats injected with ethanol alone on Day 2 or 4 were assigned to the control group. In a preliminary investigation, a solvent of 70% ethanol had no effect on luteal function of early pregnant rats. In Exp. II, rats were treated with RU 486 as in Exp. I but were killed on Day 1, 2, 3, 4, 5 or 6 to investigate the effects of RU 486 on 3 β -HSD and 20 α -HSD in CL.

Hormone assays. All blood samples were left at room temperature for 30 min. Serum was separated from blood and stored at -20°C until the assay for progesterone and oestradiol-17 β by radioimmunoassay kits obtained from International CIS (Sorin Biomedica, Saluggia, Italy). The sensitivities of the assay were 0.10 ng/ml for progesterone and 10 pg/ml for oestradiol. The cross-reactivities of the progesterone antiserum were as follows: progesterone, 100%; corticosterone, 2.5%; deoxycorticosterone, 2.5%; 17-hydroxyprogesterone, 2.0%; cortisolone, 0.25%; testosterone, 0.2%; pregnenolone, 0.16%; 20-dihydroprogesterone, 0.16%; cortisone, 0.12%; androstenedione, 0.1%; cholesterol, cortisol, dehydroepiandrosterone, oestradiol, oestriol, oestrone, etiocholanolone, 0.05%. The cross-reactivities of the oestradiol antiserum were as follows: oestradiol, 100%; oestrone, 0.7%; oestriol, 0.55%; 20-dihydroprogesterone, 0.007%; cortisol, cortisone, cortisolone, corticosterone, cholesterol, progesterone, 17-hydroxyprogesterone, etiocholanolone, pregnenolone, androstenedione, testosterone, dehydroepiandrosterone, deoxycorticosterone, 0.002%. The intra-assay variations were 7.6% for progesterone and 3.4% for oestradiol. The interassay variations were 8.5% and 9.0% respectively. All samples were assayed in duplicate.

Enzyme assays. The activities of 3 β -HSD (EC 1.1.1.51) and 20 α -HSD (EC 1.1.1.149) were assessed by using the methods reported by Kuhn & Briley (1970) and Okazaki *et al.* (1976) with a slight modification. CL were weighed and homogenized in 0.25 M-sucrose at 0°C in a glass homogenizer at a concentration of 10 mg wet weight/ml. The homogenate was centrifuged for 60 min at 105 000 *g*. The supernatant was used for the assay of 20 α -HSD activity. The precipitate was rehomogenized in the concentration stated above and centrifuged at 800 *g* for 5 min. The supernatant was used for the assay of 3 β -HSD activity. The assay mixture of 3 β -HSD contained 40 μ mol glycine-NaOH (pH 9.4), 0.9 mg bovine serum albumin, 0.5 μ mol oxidized nicotinamide adenine dinucleotide (NAD⁺) and enzyme solution (0.05 ml). That of 20 α -HSD contained 50 μ mol Tris-HCl (pH 7.5), 0.9 mg bovine serum albumin, 0.5 μ mol oxidized nicotinamide adenine dinucleotide phosphate (NADP⁺), and enzyme solution (0.05 ml). The assay mixtures were preincubated for 5 min at 37°C, and the reaction was started by the addition of the respective 3 β -HSD or 20 α -HSD substrates in a final volume of 0.80 ml. The substrate for 3 β -HSD was 0.005 mg pregnenolone (0.1 mg/ml in ethanol). That for 20 α -HSD was 0.015 mg 20 α -hydroxypregn-4-en-3-one (0.5 mg/ml in ethanol). The 3 β -HSD or 20 α -HSD activities were assayed spectrophotometrically dependent on the increase in NADH or NADPH absorbance at 340 nm respectively, and expressed as munits/mg protein. The amount of enzyme producing 1 μ mol NADH or NADPH was defined as 1 unit of enzyme activity. The method of Lowry *et al.* (1951) was used for the protein determination using bovine serum albumin as the standard.

Electron microscopic examination. For electron microscopy, the tissue specimens of corpora lutea were cut into small pieces, which were fixed in a chilled 2.5% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4) and post-fixed in 1% osmic acid. Dehydration, embedding and 1 μ m sectioning were performed as routine procedures, and ultrathin sections were stained with uranyl acetate and lead citrate, and were examined with an Hitachi H-300 electron microscope.

Statistical analysis. Statistical evaluations of the results were done with unpaired Student's *t* tests or with Tukey's tests for multiple comparison. The threshold of significance was set at *P* = 0.05.

Results

Implantation sites

As shown in Table 1, there were very few implantation sites in Group 1 and none in Group 2 rats treated with RU 486.

Ovarian steroid hormones

As shown in Table 1, progesterone and oestradiol concentrations in Group 1 rats on Day 4 differed from those of the control rats and progesterone values in Group 2 rats on Day 6 were lower

Table 1. Effect of RU 486 on the number of implantation sites and serum concentrations of ovarian steroid hormones examined on Days 4 and 6 of pregnancy in rats

	Control group		Group 1		Group 2
	Day 4	Day 6	Day 4	Day 6	Day 6
No. of implantation sites		11.2 ± 0.4 (17)		1.4 ± 0.6** (14)	0** (23)
Progesterone conc. (ng/ml)	47.0 ± 2.2 (4)	69.7 ± 10.3 (7)	27.2 ± 8.4* (5)	52.9 ± 10.9 (7)	38.3 ± 9.6 (8)
Oestradiol conc. (pg/ml)	17.5 ± 1.2 (4)	22.3 ± 2.3 (7)	29.8 ± 3.8* (5)	26.0 ± 3.3 (7)	35.5 ± 8.4 (8)
Progesterone/oestradiol ratio (× 10 ³)	2.73 ± 0.24 (4)	3.41 ± 0.75 (7)	1.04 ± 0.37* (5)	1.72 ± 0.33 (7)	1.35 ± 0.33** (8)

Values are mean ± s.e.m. for the no. of animals in parentheses.

**P* < 0.05 compared with control value on Day 4.

***P* < 0.01 compared with control value on Day 6.

Table 2. Effect of RU 486 on enzyme activity in rat corpora lutea on Day 6 of pregnancy

	3β-HSD activity (mU/mg protein)	20α-HSD activity (mU/mg protein)
Control group	10.1 ± 0.6 (11)	0 (11)
Group 1	9.8 ± 1.0 (9)	7.4 ± 3.1* (5)
Group 2	7.5 ± 0.6* (9)	17.5 ± 1.2*† (5)

Values are mean ± s.e.m. for the no. of animals in parentheses.

**P* < 0.01 compared with control value.

†*P* < 0.01 compared with Group 1 value.

than those in controls (*P* < 0.05, unpaired Student's *t* test). The progesterone/oestradiol ratio calculated from the progesterone and oestradiol values of the respective rats was lower than that of controls in all experimental animals.

Steroid converting enzyme activities on Day 6

The activity of 3β-HSD was significantly lower than that of the control rats in Group 2 rats (Table 2). There was no detectable activity of 20α-HSD in the controls, but values in Group 2 rats were twice as great as those in Group 1 rats (Table 2).

Daily activities of steroid-converting enzymes

As shown in Fig. 1(a), 3β-HSD activity rose gradually from Day 1 to 6 in the control group. In Group 1, 3β-HSD activity declined at Day 3, and then was as high as in the controls on Day 4, 5

Fig. 1. Daily changes in the activities of (a) 3β-HSD and (b) 20α-HSD in control rats, and rats in Group 1 and 2. Values are mean $\pm$ s.e.m. for the no. of animals in parentheses. † $P < 0.05$ compared with control value on Day 3; * $P < 0.01$ compared with control value on Day 6; ** $P < 0.01$ compared with Group 1 value on Day 6.

and 6. In Group 2, values were lower on Day 5, but increased by Day 6, albeit to values lower than in the control group. In contrast, 20α-HSD activity declined from Day 1 to 6 in the control group, but increased at Days 4 and Day 6 in Group 1 and 2 respectively (Fig. 1b).

Electron microscopic examination

In Group 2 rats the spaces between luteal cells were wider in Group 2 rats than in controls (Figs 2a & 2c). Lipid droplets in the cytoplasm of the control luteal cells were uniform in size and had a high electron density (Fig. 2a). In contrast, those in the cytoplasm of Group 2 CL were of various sizes with vacuoles and relatively low electron density (Fig. 2c). The nuclei of the control luteal cells were round and well-expanded (Fig. 2a), while those of Group 2 rats were irregularly shaped

Fig. 2. Electron micrographs of corpora lutea on Day 6 of pregnancy from a control rat ((a) $\times 2200$; (b) $\times 34\,000$) and a Group 2 rat ((c) $\times 2600$; (d) $\times 28\,000$).

(Fig. 2c). Smooth endoplasmic reticulum (SER) and the mitochondria of the control luteal cells were well-developed and abundant (Fig. 2b). In comparison, the SER was sparse, and elongated mitochondria were observed in Group 2 CL. Some mitochondria contained small granules with high electron density (Fig. 2d).

Discussion

In the present study, a dose of 30 mg RU 486/kg exerted an anti-implantation effect in both experimental groups. This result is in agreement with the report of Philibert *et al.* (1985). The effect was more marked in Group 2 than in Group 1. RU 486 presumably acts directly on the endometrium, thus inhibiting implantation. Since there was a significant decrease in progesterone concentration on Day 4 in Group 1, it is assumed that this decreased progesterone is responsible for the unsuitable development of the endometrium. The normality of the progesterone concentration in Group 1 on Day 6 suggests that the impairment of corpora lutea by RU 486 administered on Day 2 was temporary and that the function was fully recovered by Day 6. This speculation was supported by the fact that luteal 3β -HSD and 20α -HSD activities returned to the control level after their initial changes. The present results showed that the serum concentration of oestradiol changed significantly in Group 1 on Day 4. This raised serum oestradiol concentration may have nothing to do with RU 486 inhibiting implantation (Psychoyos & Prapas, 1987). As reported by Gidley-Baird *et al.* (1986), the progesterone/oestradiol ratio showed more significant changes than did progesterone alone, thus indicating its role as a better predictor of implantation failure than the absolute concentrations of either ovarian hormone.

The present result showed a significant decrease in the activity of the 3β -HSD, which is responsible for the conversion of pregnenolone to progesterone, indicating that RU 486 decreases progesterone production by inhibiting the 3β -HSD activity in luteal cells. The suppression of 3β -HSD by RU 486 observed on Day 3 in Group 1 rats was temporary and was followed by a progressive increase in a manner similar to that in the control rats until Day 6, resulting in a serum progesterone concentration close to the control level on Day 6 in these rats.

The enzyme 20α -HSD catabolizes progesterone to 20α -hydroxypregn-4-en-3-one and serves as a marker of luteolysis (Pupkin *et al.*, 1966; Lamprecht *et al.*, 1969; Strauss & Stambaugh, 1973). The fact that the 20α -HSD activities were increased on Day 4 and Day 6 in rats in Group 1 and 2 respectively indicates a luteolytic effect of RU 486 on the corpora lutea of pregnant rats. Unlike 3β -HSD, 20α -HSD showed the maximum changes 2 days after the drug injection. The subsequent decrease on Day 6 in Group 1 rats also reflects the recovery of the luteal cells.

To confirm the luteolytic changes indicated by the changes in serum progesterone concentration and luteal steroid dehydrogenase activities, the corpora lutea of rats in Group 2 were studied ultrastructurally. The morphological changes in these corpora lutea were characteristic for luteolysis, and consistent with the luteolytic findings observed in the corpus luteum of pregnant rats treated with abortifacient agents such as prostaglandin F-2 α or cyanoketone (Okamura *et al.*, 1972; Okamura & Yoshida, 1974).

The mechanism by which RU 486 causes luteolysis of early pregnant corpora lutea is suggested to be as follows; (1) it has a suppressive effect on the hypothalamo-pituitary axis causing decreased secretion of luteotrophic factors from the pituitary gland; (2) it has a direct chemical action on the luteal cells in pregnancy; and (3) it has antiprogestagenic activity at the level of the luteal cells. The last possibility is interesting in that lutein cells appear to need progesterone, their final product, to maintain their own cellular viability.

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