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Relationship between progesterone suppression and pregnancy in rats

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Four luteolytic agents were administered to groups of pregnant rats to examine the quantitative relationship between serum progesterone levels and the maintenance of pregnancy. Each agent inhibited progesterone in a dose-dependent manner, however only three, azastene, thiosemicarbazone and dihydrotestosterone, adversely affected pregnancy. A statistical analysis of the data suggests that, regardless of the mechanism of action of a particular luteolytic agent, a treatment-induced depression of serum progesterone to concentrations less than 45% of that of the controls on day 11 of pregnancy is incompatible with pregnancy maintenance.

Several distinct classes of compounds are currently under development as luteolytic agents with the potential application as contraceptives. These compounds include 3β-hydroxy-steroid dehydrogenase-Δ^{5-4}-isomerase (3β-HSD) inhibitors (Snyder & Schane 1985), luteinizing hormone releasing hormone (LHRH) agonists (Corbin 1979) and prostaglandins (Buckle & Nathanielsz 1973). Specific compounds of these classes have been shown effectively to lower circulating progesterone levels in-vivo although they affect progesterone synthesis and/or secretion at different levels within the luteotropic complex.

Our aim was to compare the relationship between serum progesterone levels and pregnancy during midgestation by inhibiting its secretion with various compounds which act by different luteolytic mechanisms. The compounds used were azastene, an inhibitor of 3β-HSD (Snyder & Schane 1985), dihydrotestosterone, an inhibitor of LH release (Lotz & Krause 1981), nafoxidine, a potent anti-oestrogen (Duncan et al 1963) and 5-bromo-2-thienyl ethyl ketone thiosemicarbazone, an inhibitor of luteal progesterone secretion which does not inhibit 3β-HSD (unpublished observations). The information we obtained may be used to compare the relative efficacy and potency of various luteolytic compounds even when they do not share a common mechanism of action. The results suggest that pregnancy is always terminated when serum progesterone levels are decreased to levels less than 45% of control values during midgestation. In addition, this effect is independent of the putative mechanism of action of the luteolytic agent. The information contained in this study is of particular importance in the identification of pharmacological agents with the potential to regulate fertility.

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Materials and methods

Animals. Timed-pregnant Sprague-Dawley CD rats were purchased from Charles River Breeding Laboratories (Wilmington, MA) and received on day 5 of pregnancy (day 1 = the day of a sperm positive vaginal smear). The rats were housed, 2–3/cage, in a temperature-controlled (20–22 °C) room under a 14 h light/10 h dark period (lights on 0600–2000 CST) and given free access to a commercial rat diet (Purina, St Louis, MO) and water. Pregnancy was confirmed on day 14. All rats without evidence of uterine implantation sites were excluded (never pregnant). The mean (±s.d.) number of implantation sites for the rats in this investigation was 10·8 ± 1·3 with a range of 6 to 17 sites.

Procedure. Azastene, 5-bromo-2-thienyl ethyl ketone thiosemicarbazone (TSC), dihydrotestosterone (DHT) (Sigma Chemical Co, St Louis, MO) or nafoxidine (Upjohn Co, Kalamazoo, MI) was administered subcutaneously, in a dose-response manner on days 10–13 of pregnancy at 0800 h, in corn oil at a volume of 1·0 mL kg^{-1}. Vehicle-treated rats served as controls. Doses were chosen to provide a dose range of agent likely to affect pregnancy adversely in some but not all animals. Blood samples were obtained from each rat at 0800 h on days 10 and 11 of pregnancy by orbital puncture under light ether anaesthesia. On day 14 of pregnancy each rat was decapitated and autopsied to assess the effect of treatment on pregnancy maintenance. Trunk blood, for serum progesterone analysis, was collected immediately and the uterus of each animal was examined to determine the number of normal and resorbing implantation sites. Pregnancy maintenance was expressed as the ratio of dams with one or more normal implantation sites to the total number of pregnant dams within a treatment group.

Progesterone radioimmunoassay. Blood samples were allowed to clot for at least 6 h at 4 °C, and the serum separated from the whole blood by centrifugation (2000 *g*, 4 °C, 15 min). Serum progesterone concentrations were determined by radioimmunoassay (RIA) according to Overstrom et al (1980) with two modifications.

First, the steroid was extracted from the serum by triplicate washes with 5 mL of light petroleum (b.p. 40–60 °C), and the solvent phase of each wash was decanted after rapid freezing of the aqueous layer in an acetone dry ice bath. Second, the antibody (purchased from Dr G. Niswender, Colorado State Univ., Ft Collins, CO, Lot No. 337) was used at a titre of 1 : 2020. The antibody was tested for cross-reactivity to the four agents and found to react minimally with each (azastene <0·1%; TSC <0·01%; DHT <1·0%; nafoxidine <0·1%) as determined by displacement of titrated progesterone from the antibody. Eighty, day 10 serum samples chosen at random and all other samples were analysed in duplicate. The inter- and intra-assay coefficients of variation of the RIA were 6·0 ± 1·0% and 4·0 ± 0·3%, respectively, with a sensitivity of 0·25 ± 0·05 ng mL^{-1} serum.

Statistics. Treatment-induced depressions of serum progesterone concentrations on days 11 and 14 of pregnancy were analysed by an analysis of variance, and statistical differences between doses were discerned by a Duncan's New Multiple Range Test (Steel & Torrie 1960a). A similar analysis was used to determine differences in progesterone levels among treatments in animals pooled according to pregnancy status. The effect of the treatments on pregnancy maintenance was assessed by a Mann-Whitney U-test (Freund 1973).

A 3 × 2 Chi-square analysis (Steel & Torrie 1960b), comparing the effect of three levels of serum progesterone on two levels of pregnancy status, was performed on the day 11 serum progesterone data pooled over treatments.

Table 1. Effect of azastene, thiosemicarbazone (TSC), dihydrotestosterone (DHT), and nafoxidine on days 10–13 of pregnancy in rats on pregnancy maintenance and serum progesterone concentrations.

Dose[a] (mg kg^{-1} day^{-1})	Pregnancy Maint.[b]	Foetal Viabil.[c]	Serum progesterone concn mean ± s.e.m. (ng mL^{-1})[d] on day 11	on day 14
Azastene				
0	11/11	112/118	61·9 ± 3·1	105·7 ± 5·8
2	9/9	96/100	44·5 ± 2·6*	85·9 ± 1·2
4	5/7*	36/60	30·7 ± 3·7*	53·9 ± 6·2*
8	4/7*	32/68	N.V.[e]	59·2 ± 11·8*
12	1/9**	3/91	33·5 ± 3·6**	31·2 ± 7·8**
16	0/10**	0/116	21·5 ± 2·6**	26·4 ± 4·1**
Thiosemicarbazone				
0	17/17	181/192	59·6 ± 3·2	110·6 ± 8·4
4	5/5	63/67	44·5 ± 7·2	68·1 ± 6·6*
8	3/3	37/38	52·3 ± 6·5	68·8 ± 4·1*
12	0/9**	0/109	12·9 ± 8·7**	9·3 ± 0·9*
Dihydrotestosterone				
0	16/16	176/186	62·6 ± 5·3	101·8 ± 5·9
2	7/7	67/69	63·8 ± 3·7	116·3 ± 5·9
4	7/8	65/87	62·5 ± 6·6	89·9 ± 19·8
8	4/7*	40/81	57·8 ± 4·6	80·2 ± 16·7
16	5/8*	46/85	40·5 ± 6·6*	84·0 ± 11·2*
24	5/9*	47/95	35·4 ± 9·6*	51·2 ± 10·8**
32	5/8*	46/86	37·4 ± 7·0*	65·1 ± 12·2*
Nafoxidine				
0	12/12	123/133	59·0 ± 2·2	87·2 ± 6·4
0·03	6/6	46/50	50·1 ± 3·3*	76·3 ± 5·3
0·30	9/9	105/110	50·4 ± 3·3*	46·6 ± 3·9*
3·00	5/5	40/43	41·5 ± 3·1*	34·1 ± 5·1**

[a] Once daily subcutaneously from day 10 through day 13 (day 1 — the day of finding sperm in the vaginal smear).
[b] No. of dams with at least 1 normal implantation site/total no. of dams.
[c] No. of normal implantation sites/total no. of implantation sites.
[d] Pretreatment progesterone levels (mean ± s.e.m.) for a random selection of 20 rats from each study was 51·9 ± 2·5 ng mL^{-1}.
[e] Sera in sufficient quantities for progesterone assay were obtained from only 3 dams, all of which maintained pregnancy. The group mean ± s.e.m. (54·8 ± 2·5 ng mL^{-1}) was considered invalid for this analysis.
* Significantly different from the control group ($P < 0·05$).
** Significantly different from the control group ($P < 0·01$).

Results

The effects of the four agents used in the present study on serum progesterone levels and pregnancy maintenance are shown in Table 1. Although each agent dose-dependently depressed progesterone levels relative to day 11 control values, pregnancy was compromised in rats treated with azastene, DHT and TSC but not in rats treated with nafoxidine.

Table 2 presents an examination, by an analysis of variance, of the pooled serum progesterone levels of animals in which the treatments terminated pregnancy. The analysis revealed that the progesterone levels do not differ ($P > 0·05$) among the treatments on day 11 of pregnancy. A similar effect was noted among animals in which the treatments failed to terminate pregnancy. Progesterone levels on day 14, however, are shown to differ ($P < 0·05$) among treatments, as well as pregnancy status.

The 3 × 2 Chi-square evaluation of serum progesterone data from all treated animals on day 11 of pregnancy (Table 3) reveals that pregnancy always terminated when the progesterone concentrations were depressed to less than 45% of the corresponding control values. Depression to levels between 45 and 55% of the controls resulted in partial pregnancy maintenance. Serum progesterone levels that are at least 55% that of the control values are fully compatible with maintenance of pregnancy. These estimates are independent of the mechanism of action of each respective inhibitor.

Table 2. Serum progesterone levels (mean ± s.e.m.) in rats terminating pregnancy and in rats maintaining pregnancy pooled over all doses of the progesterone synthesis inhibitors.

Pregnancy termination Day 11	Day 14	n	Pregnancy maintenance Day 11	Day 14	n
Azastene					
26·2 ± 3·1[a]	30·5 ± 5·2[a]	20	37·5 ± 5·3[c]	53·1 ± 6·6[c]	20
Thiosemicarbazone					
12·9 ± 8·7[a]	9·3 ± 0·9[b]	9	48·4 ± 4·7[c]	68·3 ± 4·0[c]	8
Dihydrotestosterone					
27·9 ± 8·7[a]	26·3 ± 8·4[a,b]	14	52·5 ± 4·0[c]	98·7 ± 6·8[d]	33
Nafoxidine					
—	—	0	48·1 ± 2·8[c]	52·4 ± 4·6[c]	20

Progesterone levels in groups with different superscripts within columns and within rows are statistically different ($P < 0·05$).

Table 3. The effect of different levels of serum progesterone on pregnancy analysed by a 3 × 2 Chi-square. Day 11 progesterone levels were used in the analysis. The contribution of individual cells to the overall Chi-square is in parentheses within each.

Serum progesterone (% of control)	Pregnancy status: Terminated (n)		Maintained (n)	
>55%	Observed	0·0	Observed	37·0
	Expected (13·04)**	13·0	Expected (7·10)**	24·0
45–55%	Observed	15·0	Observed	42·0
	Expected (1·29)	20·1	Expected (0·70)	36·9
<45%	Observed	28·0	Observed	0·0
	Expected (33·31)**	9·9	Expected (18·13)**	18·1

Overall Chi-square = 73·57** with 2 df.
** $P < 0.01$.

Discussion

The results shown in Table 1 demonstrate an unequivocal dependence of pregnancy maintenance on an adequate level of progesterone during midgestation in rats. These data are not unique in that this relationship has been well-established and amply documented in the literature (for review see Rothchild 1981). However, our study does provide comparative data for four compounds which act as luteolytic agents by different mechanisms, including the first evidence of functional luteolysis in pregnant animals following treatment with TSC and nafoxidine. This study, however, has established that there is a critical level of circulating progesterone for pregnancy maintenance on day 11 of pregnancy in rats. These data provide a convenient and quantifiable endpoint for the assessment of new compounds, an endpoint which appears to be independent of the mechanism of induced luteolysis.

The pharmacological control of progesterone synthesis can be achieved at many levels. During midgestation in rats luteotropic influences include rat placental lactogen (Blank et al 1979), luteinizing hormone and prolactin (Rothchild 1981). These hormones may affect progesterone synthesis directly (Terranova & Greenwald 1981) as well as through an indirect mechanism involving oestradiol-17β (Gibori et al 1978). As such, treatments which inhibit the production and/or secretion of these luteotropins will adversely affect pregnancy. In addition, experimental manipulations which compromise pregnancy by a direct effect on the foetuses, also depress progesterone levels (Ochiai & Rothchild 1981). The present study provides a means to assess comparatively the contragestational activity of a variety of luteolytic agents. As we have shown, the measurement of serum progesterone levels on day 11 accurately reflects pregnancy status and thus this endpoint can be used to measure the potency and efficacy of agents which act by differing luteolytic mechanisms. The measurement of circulating levels of other hormones, such as gonadotropins, releasing factors, oestradiol-17β and testosterone, following treatment with peptide-hormone agonists (Yamazaki 1984; Sridaran 1986), antisera to releasing hormones (Terranova & Greenwald 1981), and anti-oestrogen (Rodway & Rothchild 1980) has yielded contradictory data. These hormones, therefore, cannot be measured to predict the efficacy of novel luteolytic compounds reliably.

In the present study, the measurements of progesterone levels on day 14 of pregnancy is not as reliable an indicator of compound efficacy. For example, progesterone levels on day 14 after tretment with 12 mg kg^{-1} azastene are depressed 70·5%, yet pregnancy is not terminated in all animals in the group.

In summary, our results indicate that the suppression of serum progesterone to levels less than 45% of the controls on day 11 is incompatible with the maintenance of pregnancy. In addition, suppression to levels between 45 and 55% of controls results in pregnancy maintenance in some, but not all, animals, and levels greater than 55% had no adverse effect on pregnancy maintenance. The effect was independent of the mechanism of action of the luteolytic agent, however, discrete drug effects on serum progesterone levels were seen on day 14. Thus, serum progesterone levels on day 11 appear to be a convenient endpoint for assessing the luteolytic and contragestational potential of pharmacological compounds.

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Interactions of trimebutine with guinea-pig opioid receptors

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Affinities of trimebutine (TMB) and *N*-desmethyl trimebutine (NDTMB) for mu, delta and kappa opioid receptor subtypes have been examined using specific ^{3}H-ligands and guinea-pig membrane. TMB and NDTMB showed a relative higher affinity for the mu receptor subtype although they were, respectively, 30-and 48-fold less active than morphine. The receptor selectivity index for mu, delta and kappa were 100 : 12 : 14·4 for TMB, 100 : 32 : 25 for NDTMB and 100 : 5 : 5 for morphine. The sodium shift ratio was 14 for TMB, 10 for NDTMB and 37 for morphine. These data show that (unlike morphine, a pure mu agonist) TMB and NDTMB can be classified as weak opioid agonists and confirm that peripheral opioid receptors mediate their gastrointestinal motility effects.

Trimebutine (2-dimethylamino-2-phenylbutyl 3,4,5-trimethoxybenzoate hydrogen maleate) (TMB) is used in the treatment of various digestive tract disorders including dyspepsia, abdominal pain, irritable bowel syndrome (Moshal & Herron 1979; Luttecke 1980) and postoperative ileus (Malavaud 1972). Studies have shown that TMB stimulates small intestine motility by inducing regular spike activity in man (Grandjouan et al 1985, Valori et al 1985) and dog (Fioramonti et al 1984; Poitras et al 1985). The stimulatory effects of TMB are blocked by previous intravenous administration of naloxone, but not after intracerebroventricular (i.c.v.) injection of the mu antagonist, and are not reproduced after i.c.v injection of the drug. These results suggest that TMB acts on the small intestine through peripheral opioid receptors (Fioramonti et al 1984).

Moreover, recent work (Blanquet et al 1985) has shown that TMB induces a naloxone-sensitive spiking activity in cat and rabbit colon.

Since no data have been reported so far on the direct interaction of TMB and *N*-desmethyl trimebutine (NDTMB), its main metabolite, with opioid receptors, we have investigated the in-vitro affinity of both compounds for mu, delta and kappa opioid receptor subtypes using guinea-pig brain membranes and specific ligands.

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Methods

Membrane preparation. Male tri-coloured guinea-pigs (30–325 g) were decapitated, brains rapidly removed, and the cerebellum discarded. The brains were then chilled in cold 0·05 M Tris buffer (pH = 7·5). Membranes for binding assays were prepared according to Kosterlitz et al (1981). Briefly, the brain tissue was homogenized in Tris buffer 0·05 M (pH = 7·5 at 0 °C), centrifuged at 49 000 *g* for 10 min, the pellet resuspended in Tris buffer, incubated at 37 °C for 45 min and centrifuged again. After a last washing of the pellet, the final homogenate was used for binding assays.

Binding assay. Aliquots of homogenate suspension (0·1 mL corresponding to 100 mg brain tissue), cold ligands and tritiated ligands (total volume 1 mL) were incubated for 40 min at 25 °C, filtered through Whatman GF/B glass fibre filter discs and washed three times with 4 mL ice-cold Tris buffer solution. Specific binding corresponded to the difference between total binding and non-specific binding obtained by addition of high concentration of levallorphan (10 μM).

The following ligands (New England Nuclear Corp) were used at the indicated final concentration: [^{3}H]dihydromorphine ([^{3}H]DHM: 73·6 Ci mmol^{-1}), 0·7 nM; [^{3}H]naloxone ([^{3}H]NAL: 42·7 Ci mmol^{-1}), 1 nM; [^{3}H]D-ala^2-D-leu^5-enkephalin ([^{3}H]DADLE: 46·9 Ci mmol^{-1}), 0·5 nM and [^{3}H]ethylketocyclazocine ([^{3}H]EKC: 18·7 Ci mmol^{-1}), 0·5 nM.

Calculations. The shift of the competition curves with [^{3}H]NAL by 100 mM NaCl was used to determine whether TMB and NDTMB behave as agonist or antagonist according to Pert & Snyder (1974). The equilibrium dissociation constant (KD) and the maximum number of binding sites (B_{max}) were determined by Scatchard analysis of saturation curves.

IC50 were determined according to the Hofstee method from the least squares linear regression curves of log(B/Be − B) versus log I where: I was the concentration of test compound in mol L^{-1}, Be the total