PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 170–174 (1979)

Inhibition of Ovulation in Hamsters by the Protein Synthesis Inhibitors Diphtheria Toxin and Cycloheximide (40639)

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Pool and Lipner (1) reported that actinomycin D, an inhibitor of DNA-directed synthesis of messenger RNA (transcription), inhibited ovulation in rabbits. In this species, coitus triggers the release of pituitary luteinizing hormone (LH) into the blood stream and ovulation occurs about 11 hr later. These workers injected the inhibitor directly into ovarian follicles and observed inhibition of ovulation only by injections given up to 5 hr after mating (1). Similar results were obtained with puromycin and cycloheximide (2), inhibitors of mRNA-directed protein synthesis (translation). These findings led Pool and Lipner (2) to suggest that RNA and protein synthesis essential for ovulation is restricted to the early part of the 11-hr ovulatory process initiated by coital-induced LH release. Barros and Austin (3) reported that systemic injection of actinomycin D inhibited ovulation in the golden hamster, a spontaneously ovulating species. In this paper we report effects of systemic injection of two translational inhibitors, diphtheria toxin and cycloheximide, on ovulation in hamsters. Diphtheria toxin depletes cells of elongation factor 2, which is needed for growth of nascent peptide chains, by catalyzing its ribosylation by nicotinamide adenine dinucleotide (4). Cycloheximide prevents both peptide chain initiation and elongation by interacting with the 60S ribosomal subunit (5).

Materials and methods. Golden hamsters (*Mesocricetus auratus*) were used. In this species, ovulation occurs every fourth day and is photoperiodically controlled. When the laboratory is illuminated daily for from 12 to 16 hr, and when the midpoint of this photoperiod is used as the noon reference, the hypothalamic–pituitary system releases LH into the blood stream at about 2:30 PM on Day 4. The released LH stimulates the ovaries to secrete progesterone, which initiates behavioral estrus at about 5:00 PM, and further action of the LH causes follicular rupture (ovulation) about 1:30 AM of the next day (Day 1), i.e., about 11 hr after LH release. Therefore, drugs that block ovulation when injected before but not after 2:30 PM on Day 4 may be assumed to act on the CNS–pituitary system, whereas drugs effective after 2:30 PM may be assumed to act directly on the ovaries (6–12).

Hamsters from our own closed colony (FDA strain) or from the Lakeview colony (LVG:LAK strain) were used. They were fed Purina chow and water. The experimental room was illuminated daily from 4:00 AM to 8:00 PM E.S.T. (i.e., light–dark (LD) 16:8 with the midpoint of the light period synchronized with solar noon). FDA hamsters were born and raised in the experimental room. Lakeview hamsters were acclimated in it for at least 1 month before experimentation.

Materials were injected i.p. as a single dose in a volume of 5 ml/kg of body weight at specific times on Day 4, which was identified by daily analysis of vaginal washings (12). The hamsters were killed with ether or chloroform on the next day (Day 1) between 6:45 AM and 4:30 PM. Fallopian tubes were excised and ova were counted under a microscope at 100× as each tube was pressed separately between two glass slides. Data were recorded as the combined number of ova from both tubes of an individual hamster. Normally, 6–16 (mean value 12) ova are ovulated (6).

Diphtheria toxin was prepared as previously described (13) and had a potency of 8.8 guinea pig minimal lethal doses (MLD)/μg of toxin protein. Toxin was dialyzed at 4° in potassium phosphate buffer (0.01 *M*, pH 6.9) and bovine serum albumin was added to a concentration of 0.05%. The final solution, containing 570 μg of toxin/ml, was stored at −4° until the time of injection when it was diluted with buffer to the desired doses. Equine diphtheria antitoxin (Wyeth Co.,

0037-9727/79/090170-05$01.00/0

Philadelphia, Pa.) was diluted with the phosphate buffer. Cycloheximide (Acti-Dione, Calbiochem Inc., San Diego, Calif.) and sodium phenobarbital were dissolved in physiological saline.

Results. The LD_{50} of diphtheria toxin in hamsters was 6.5 μg/kg of body weight; confidence limits were 4.8–8.7 μg/kg. The MLD was = 11 μg/kg, or 1 μg/hamster. Since the potency of the sample was approximately nine guinea pig MLD/μg, it is estimated that hamsters are nine times less sensitive to diphtheria toxin than are guinea pigs.

Diphtheria toxin was found to be a potent inhibitor of ovulation in hamsters. Data in Table I show that a dose of two hamster MLD (22 μg/kg) inhibited ovulation completely in all hamsters when injected between 1:30 and 4:00 PM on Day 4 of the estrous cycle. Thereafter the toxin injection was gradually less effective, and was completely ineffective by 7:30 PM of the same day. Inhibition of ovulation by toxin was eliminated by diphtheria antitoxin, and thus it can be concluded that the blocking effect on ovulation is toxin specific. Dose–response data of the inhibitory effect of diphtheria toxin on ovulation are shown in Table II. The block in ovulation was found to be dose dependent. Ovulation was inhibited when the dose administered at 7:30 PM was increased to 4 and 8 MLD.

TABLE I. EFFECT OF DIPHTHERIA TOXIN ON OVULATION IN HAMSTERS: TIME RESPONSE

Time of i.p. injection on Day 4 of cycle[a] (PM)	Tubal ova counts on the day after injection[b]		
	Toxin dose		
	2 MLD[c] (22 μg/kg)	2 MLD + 200 units antitoxin/kg	Controls (5 ml buffer/kg)
1:30	0, 0, 0, 0, 0, 0	10, 11, 11	
2:30	0, 0, 0, 0, 0, 0	11, 11, 12	
3:30	0, 0, 0, 0		
4:00	0, 0, 0, 0, 0, 0		
4:30	0, 0, 0, 2, 4, 9	7, 8, 11, 11, 13, 14	7, 9, 14, 15
6:30	0, 0, 4, 10, 15, 16		
7:00	0, 12, 12, 14, 14, 16		
7:30	6, 9, 10, 11, 11, 13		

[a] Pituitary LH is released into the circulation at about 2:30 PM on Day 4 and ovulation occurs about 11 hr later.
[b] Each value is the total number of ova found in both fallopian tubes of an individual hamster. Normally 6 to 16 ova (mean 12) are ovulated.
[c] MLD is the minimum lethal dose for a hamster.

Cycloheximide also inhibited ovulation in hamsters (Table III). In lethality tests, doses of 80, 60, 40, and 20 mg/kg killed 4/4, 6/15, 1/8, and 0/6 hamsters within 1–6 days. Like the 2 MLD dose of diphtheria toxin, the 60 mg/kg dose of the drug inhibited ovulation in hamsters injected at 1:30 and 4:00 PM but not at 7:30 PM on Day 4. The 80 mg/kg dose of drug injected at 7:30 PM inhibited ovulation to the same extent as the 8 MLD dose of toxin (Table II). It is noteworthy that ovulation was inhibited by injection of cycloheximide as late as 10:45 PM, i.e., between 8 and 9 hr after LH release, and 2–3 hr before ovulation.

Although injection of the 60 mg/kg dose of cycloheximide or the 2 MLD dose of toxin at 1:30 or 4:00 PM on Day 4 inhibited ovulation, it did not inhibit behavioral estrus, as evidenced by an immediate display of lordosis by the treated female when exposed to a male at 7:30 PM on the day of injection or by the presence of sperm in the vaginal washing taken on the following morning.

That LH release occurred on Day 4 before 3:00 PM under the conditions of these experiments was confirmed by use of phenobarbital, an established central blocking agent for LH release (8, 11). Data in Table IV show that ovulation generally was inhibited by injection of phenobarbital at 1:30 or 1:45 but not at 3:00 PM, when injection of either toxin or cycloheximide was highly inhibitory. In a

TABLE II. EFFECT OF DIPHTHERIA TOXIN ON OVULATION IN HAMSTERS: DOSE–RESPONSE

Time of i.p. injection on Day 4 of cycle (PM)	Tubal ova counts on the day after injection				
	Controls (5 ml buffer/kg)	Toxin dose (MLD)			
		1	2	4	8
1:30	9, 9, 11, 12, 13, 16	0, 0, 0, 0, 0, 0, 1, 6	0, 0, 0		
3:30		0, 0, 1, 2, 5, 9, 11, 12	0, 0, 0, 0, 5		
7:30			7, 7, 10, 11, 11, 12	3, 7, 8, 8, 9, 9, 9	0, 0, 0, 0, 5, 6

TABLE III. EFFECT OF CYCLOHEXIMIDE ON OVULATION IN HAMSTERS

Time of i.p. injection on Day 4 of cycle (PM)	Tubal ova counts on the day after injection			
	Cycloheximide (mg/kg)			
	80	60	40	20
1:30	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 2	4, 7, 9, 10	9, 12
4:00	0, 0, 0, 0, 0, 0	0, 0, 0, 0		
6:00	0, 0, 0, 0, 6			
7:30	0, 0, 0, 3, 7, 7	5, 8, 10, 10, 12, 15		
8:30	0, 0, 1, 4, 8, 9			
10:45	0, 0, 0, 0, 1, 1, 6, 6, 8			

TABLE IV. EFFECT OF PHENOBARBITAL, DIPHTHERIA TOXIN, AND CYCLOHEXIMIDE ON OVULATION IN HAMSTERS

Time of i.p. injection on Day 4 of cycle (PM)	Tubal ova counts on the day after injection				
	Phenobarbital (140 mg/kg)	Diphtheria toxin (2 MLD)	Cycloheximide (80 mg/kg)	Solvent controls (5 ml/kg)	
				Saline	Buffer
1:30	0, 0, 0, 0, 0				
1:45	0, 0, 0, 0, 13				
3:00	0, 6, 9, 10, 11, 13, 14, 15	0, 0, 0, 0, 0, 0, 0, 15	0, 0, 0, 0, 0	9, 10, 11, 14	10, 12, 14, 15

previous report from this laboratory, the estimated time ± SE at which injection of phenobarbital blocked ovulation in 50% of a group of 346 hamsters exposed to LD 16:8 was found to be 2:30 PM ± 2 min (12), which is at the same time blood concentrations of LH rise on Day 4 in untreated hamsters exposed to LD 14:10 (9, 11).

Ovaries of hamsters providing tubal ova counts in Table IV were examined microscopically. The absence of tubal ova following injection of phenobarbital, toxin, and cycloheximide was associated with large unruptured ovarian follicles that, when pricked with a needle and observed at 30× magnification or pressed between two slides and observed at 100× magnification, released an oocyte surrounded by granulosa cells. In contrast, tubal ova were associated with large ruptured hyperemic follicles (corpora lutea) lacking oocytes. Other hamsters (two/group) were injected on Day 4 with phenobarbital (140 mg/kg) at 1:45 PM, diphtheria toxin (2 MLD) at 3:30 PM, or cycloheximide (80 mg/kg) at 3:30 PM and were killed on the following morning. None of the fallopian tubes contained ova. Ovaries were fixed in neutral-buffered formalin, serially sectioned, and stained with hematoxylin and eosin. Large unruptured and nonluteinized follicles with intact oocytes were observed in each group. Follicles of hamsters in which ovulation was inhibited by phenobarbital and cycloheximide were similar in the histologic appearance of their oocytes, and their theca and granulosa cells, which displayed various stages of mitosis. In contrast, in those in which ovulation was inhibited by toxin, the follicles contained shrunken oocytes and nondividing pyknotic granulosa cells in all phases of necrosis while theca cells appeared normal.

Discussion. The fact that ovulation was inhibited by injection of diphtheria toxin or cycloheximide after 2:30 PM on Day 4 of the estrous cycle, when release of LH from the pituitary gland into the blood stream occurs, indicates that these agents act directly on the ovaries. Since timing of the injection determines the described effects of ovulation, the effect is not secondary to the general inhibition of protein synthesis that culminates in death. The 2 MLD dose of diphtheria toxin and the 60 mg/kg dose of cycloheximide inhibited ovulation in virtually all hamsters injected up to 4:00 PM and in no hamster injected at 7:30 PM or later (Tables I–IV). Thus, the period of inhibition extends up to 5 hr after LH release. This period is remarkably similar to that obtained by Pool and Lipner (1, 2) after intrafollicular injection of actinomycin D, cycloheximide, and puromycin in rabbits (5–6 hr) and by Barros and Austin (3) after i.p. injection of actinomycin D in hamsters (4.5–6.5 hr, according to our analysis of their data). The latter workers exposed hamsters to light daily from 0800 to

2200 hr (i.e., 5:00 AM–7:00 PM if the midpoint of the photoperiod is considered noon, as in this paper) and assumed LH was released at 1400 hr (11:00 AM), when it is now known to be released 3.5 hr later (6–12). Contrary to the conclusion of Barros and Austin (3), there appears to be no significant species difference between the rabbit and hamster in this early period of susceptibility to actinomycin D. Thus, in both a reflex and a spontaneously ovulating species, injections of transcriptional and translational inhibitors delimited an almost identical period of inhibition of ovulation, extending with decreasing effectiveness over the first 5 hr of the 11-hr ovulatory process initiated by LH release. It is highly unlikely that this singular effect resulted from actions of these agents other than the one they have in common, i.e., inhibition of protein synthesis.

Pool and Lipner (1, 2) interpreted this effect as evidence that protein synthesis essential for ovulation is restricted to the first few hours after LH release. Although our results with the 2 MLD dose of toxin and the 60 mg/kg dose of cycloheximide are consistent with this suggestion, we find that higher doses are highly inhibitory when injected at 7:30 PM and later (Tables II, III). The observation that ovulation can be inhibited by cycloheximide injected as late as 8–9 hr (10:45 PM) after LH release suggests that protein synthesis vital for the process of ovulation continues throughout the 11-hr ovulatory period and is not restricted to the period immediately following LH release.

The present finding with diphtheria toxin is the first case of a protein inhibiting ovulation by direct action on the ovaries. The diphtheria toxin molecule resembles LH in containing one subunit that combines with specific receptors on the susceptible cell plasma membrane and another subunit metabolically active inside the cell (4, 14). However, it is unlikely that the toxin inhibits ovulation by occupying receptor sites for LH on ovarian cell membranes. Ovulation in hamsters can be blocked by injection of LH antiserum on Day 4 at 1:00 PM but not at 3:00 PM (8). Apparently, LH binding to ovarian receptors is complete by 3:00 PM, and yet ovulation was inhibited by diphtheria toxin injected more than 4 hr later.

Rondell (15) proposed that LH-induced ovulation involves two stages of protein synthesis: LH-induced steroidogenesis from acetate or cholesterol to progesterone, and progesterone-induced synthesis of collagenase in the ovarian follicle wall, causing its dissolution and culminating in ovulation. Strickland and Beers (16, 17) suggested that the lytic enzyme is not collagenase but plasmin, converted from plasminogen by the enzyme, plasminogen activator. In any case, the fact that diphtheria toxin and cycloheximide, as well as actinomycin D (3), inhibited ovulation but not behavioral estrus, which requires progesterone, favors the idea that these agents inhibit synthesis of enzymes responsible for dissolution of the follicle walls rather than enzymes involved in steroidogenesis.

Summary. Diphtheria toxin and cycloheximide, structurally unrelated translational inhibitors of protein synthesis, inhibited ovulation in hamsters. Inhibition resulted from i.p. injection given well after the 2:30 PM release of pituitary luteinizing hormone (LH) that initiates the 11-hr ovulatory process. Thus it may be concluded that these agents inhibit ovulation by direct action on the ovaries. Both luteinization and follicular rupture were inhibited. Follicles inhibited by cycloheximide were similar histologically to control follicles prevented from ovulating by central blockade of LH release with phenobarbital, whereas follicles inhibited by toxin contained shrunken oocytes and necrotic granulosa cells. Low doses of both agents inhibited ovulation only when injected within 5 hr after LH release. However, high doses were effective at later times. Similar ovulation inhibition responses to both the microbial toxin and cycloheximide at high doses suggest that protein synthesis essential for ovulation occurs throughout the 11-hr ovulatory process initiated by LH and is not restricted to the early part of this period.

We thank Dr. Louis Kasza (Food and Drug Administration) for histological analysis of the sectioned ovaries.

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Received July 5, 1978. P.S.E.B.M. 1979, Vol. 162.