Blockage of ovulation in the explanted hamster ovary by a collagenase inhibitor

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Summary. Hamster ovaries explanted at 21:00 h on the day of pro-oestrus were incubated with talopectin, a potent microbial inhibitor of collagenases, until 10:30 h on the next morning. A talopectin concentration of 0.16 mM completely blocked ovulation *in vitro*. The blocking effect decreased markedly in ovaries excised at 23:00 h or later. The results suggest that proteolysis by collagenases is indispensable for the ovulatory process in the hamster.

Introduction

There is considerable evidence implicating collagenolytic enzymes in the ovulatory process of mammalian and avian ovaries (Nakajo, Zakaria & Imai, 1973; Espey, 1974, 1980; Bjersing & Cajander, 1975; Okamura, Takenaka, Yajima & Nishimura, 1980; Fujii, Tojo & Koga, 1981). Collagenolytic enzymes have been detected in the ovarian follicles of rabbits, sows, women and rats (Espey & Rondell, 1968; Espey & Coons, 1976; Morales, Woessner, Howell, Marsh & LeMaire, 1978; Fukumoto, Yajima, Okamura & Midorikawa, 1981). However, there is no direct evidence substantiating involvement of collagenases in the ovulatory process. Murao, Katsura, Fukuhara & Oda (1980) have isolated a potent specific inhibitor of metallo-proteinases, MK-I, from a new strain of *Streptomyces*, and the inhibitor was recently named talopectin (Fukuhara, Murao, Nozawa & Hatano, 1982). In addition we have developed an in-vitro method to ovulate subnormal numbers of ova from hamster ovaries (Morioka & Ichikawa, 1982). This study was conducted to confirm the hypothesis of collagenase involvement in ovulation by examining the blockage of ovulation in the excised hamster ovary by talopectin.

Materials and Methods

Animals. Adult laboratory bred golden hamsters, *Mesocricetus auratus*, 60-90 days of age and weighing 80-150 g, were used. Animals were housed at $24 \pm 2^\circ\text{C}$ under a lighting schedule of 14 h light (05:00-19:00 h). Vaginal smears were examined every morning. Only females which showed more than 3 consecutive 4-day cycles were used. In these conditions the hamsters ovulated 12.3 ± 0.4 ova (mean $\pm$ s.e.m., $n = 15$), and ovulation occurred between 00:00 and 02:00 h on the day of oestrus which is characterized by a conspicuous vaginal discharge (Morioka & Ichikawa, 1982).

Incubation of the ovary. Animals were killed by decapitation on the day of pro-oestrus at specified times ± 15 min between 21:00 and 24:00 h. All ovaries explanted at 21:00 h or later on the day of pro-oestrus ovulated a mean number of 4.6-4.7 ova but those excised at 19:00 h or earlier rarely ovulated (Morioka & Ichikawa, 1982). The ovaries were placed in Petri dishes filled with Hanks' solution (Hanks & Wallace, 1949). Fat, ovarian bursa and oviduct were removed

aseptically. The ovaries were cultured by the procedure described by Morioka & Ichikawa (1982). Briefly the ovaries were rinsed in a fresh culture medium and placed separately on a stainless-steel wire table (2.2 mm in height) in each culture vessel which contained 2.5 ml culture medium. A Petri dish of 3.5 cm i.d. was used as the culture vessel. A volume of 2.5 ml medium had a depth of 2.6 mm in the vessel, and the surface of the table was 0.4 mm lower than the fluid level. Since the mean $\pm$ s.e.m. diameter of the ovaries was 2.3 ± 0.0 mm ($n = 10$), they were cultivated at the surface of the medium. It took about 4 min for each ovary to be excised and immersed in the culture medium. The loaded culture vessels were placed in a plastic culture chamber and immediately gassed with 95% O₂:5% CO₂ for 15 min at the rate of 1.5 litres per min. The humidity of the chamber room was maintained by placing wet cotton on the bottom of the chamber. The culture chamber was housed in an incubator at 37°C. The ovaries were incubated until 10:30 h the following day. Calf serum was then added to the culture medium to prevent adhesion of the ova to the vessel wall. The final concentration of calf serum was 15%. The vessel was gently shaken by hand to free the ova from the vessel wall. The ova shed in the medium were counted under a dissecting microscope.

Chemicals. The basic medium was Medium 199 (Nissui, Co., Tokyo) supplemented with 3.0 mg glucose, 0.1 mg L-glutamine, 0.05 mg ascorbic acid, 0.035 mg insulin, 1.9 mg sodium bicarbonate, 100 units of penicillin and 0.12 mg streptomycin per ml; the osmolarity was 270 mosmol. Talopeptin (6-deoxy- α -L-talopyranosyloxypospho-L-leucyl-L-tryptophan, MK-I) was isolated from culture filtrates of *Streptomyces mozumensis* MK-23. The inhibitor was dissolved in saline (9 g NaCl/l) at a concentration of 0.5, 2, 4 and 8 mM. The culture medium was prepared by adding 0.4 ml of the inhibitor solution to 10 ml of the basic medium. Control ovaries were incubated in the same basic medium to which 0.4 ml saline was added in place of the inhibitor solution.

Results

Dose of talopeptin for ovulatory blockage in the explanted hamster ovary

As shown in Table 1, talopeptin at a concentration of 0.02 mM significantly decreased the average number of ova ovulated per ovary. At concentrations of 0.16 and 0.32 mM only one ovum was ovulated from the 20 ovaries examined.

Table 1. Effect of talopeptin concentration on ovulation of hamster ovaries explanted at 21:00 h on the day of pro-oestrus

Talopeptin conc. (mM)	No. of ovaries examined	No. of ovaries ovulating	No. of ova ovulated	Mean $\pm$ s.e.m. (range) no. of ova/ovary
0	10	10	45	4.5 $\pm$ 0.3 ^a (3–6)
0.02	10	10	30	3.0 $\pm$ 0.3 ^b (2–5)
0.08	10	5	8	0.8 $\pm$ 0.3 ^c (0–2)
0.16	12	0	0	0
0.32	8	1	1	0.1 $\pm$ 0.1 ^c (0–1)

Means with different superscripts differ significantly, $P < 0.01$.

Time required by talopeptin to block ovulation in the explanted ovary

As shown in Table 2, 0.16 mM-talopeptin completely blocked ovulation from 14 of 16 ovaries which were explanted at 21:00 and 22:00 h on the day of pro-oestrus. The inhibitory effect of talopeptin markedly decreased when the ovaries were explanted at 23:00 and 24:00 h.

Table 2. Effect of 0.16 mM-talopeptin on ovulation of hamster ovaries explanted between 21:00 and 24:00 h on the day of pro-oestrus

Time of explantation (h)	No. of ovaries examined	No. of ovaries ovulating	Total no. of ova ovulated	Mean $\pm$ s.e.m. (range) no. of ova/ovary
21:00	8	0	0	0
22:00	8	2	3	0.4 $\pm$ 0.3 ^a (0-2)
23:00	8	8	22	2.8 $\pm$ 0.5 ^b (1-5)
24:00	8	8	25	3.1 $\pm$ 0.5 ^b (1-5)

Means with different superscripts differ significantly, $P < 0.01$.

Discussion

Since talopeptin strongly inhibits metallo-proteinases and slightly inhibits carboxypeptidase, but does not inhibit trypsin, chymotrypsin, papain or pepsin (Murao *et al.*, 1980), the present results indicate that collagenolysis is one of the indispensable factors in the ovulatory process in the hamster ovary. Ovulation blockage by talopeptin in the explanted ovary decreased markedly when the ovary was excised at 23:00 h on the day of pro-oestrus, but the integrity of the apex of the Graafian follicle at this time was still maintained. Since ovulation begins *in vivo* at 00:00 h or later on the day of oestrus, collagenolytic degradation of the follicle wall may have reached by 23:00 h a critical stage that inevitably allowed the follicles without collagenase to rupture, or other physiological event, such as proteolytic degradation of a tissue matrix other than collagenous framework may follow the collagenolysis before rupture. These must be evaluated in future studies.

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