

Inhibitory Effects of Steroid-Free Porcine Follicular Fluid on Ovulation in Rabbits

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INTRODUCTION

Our previous report¹⁾ suggested that subcutaneous injections of charcoal-treated porcine follicular fluid (CTPFF) at successive 8- or 12-hour intervals for about 5 days completely suppressed ovulations in mated rabbits, and that treatments of CTPFF at 12-hour intervals for about 5 days, followed by dosage of 10 IU of human chorionic gonadotropin (hCG), caused reduction in both numbers of ovulatory does and ovulations in ovulated does. However, dosage of 5 or 15 IU of hCG to does treated with CTPFF once a day for 10 days did not alter either the number of ovulatory does or of ovulations. Therefore, the inhibitory effect of CTPFF on ovulation seemed to be stronger with repeated treatments at 8- or 12-hour intervals than in does which received it once a day. Vulvar swelling and coloration had receded 1 or 2 days after the beginning of CTPFF treatments, and had appeared 2 days after the end of treatment.

The present study further clarified the inhibitory effect of CTPFF on ovulation caused by various dosages of hCG and on follicle-stimulating-hormone (FSH) levels in blood plasma during CTPFF treatments in rabbits.

MATERIALS AND METHODS

Animals

Fifty-eight virgin female adult Japanese White rabbits, weighing 2.5 - 4.4 kg, were used. All of the animals were reared in individual cages for over one month after purchase from a local commercial rabbit breeder, with food and water supplied *ad libitum*. They were divided into 3 groups: 10 does for treatment A, 5 for treatment B, 10 for control A, and 6 for control B in group 1; 6 for treatment and 8 for control in group 2; 6 for treatment and 7 for control in group 3 (Table 1).

Porcine follicular fluid

Porcine ovaries were collected at a local meat-packing plant, immediately placed on ice and transported to our laboratory. Follicular fluid (FF) was aspirated mainly from small and medium follicles of ovaries, excluding ovaries having corpora lutea or cystic

follicles. The fluid was pooled in a plastic bottle and stored in a freezer at -20°C until use. The frozen FF was slowly melted in a refrigerator at 4°C . To the melted FF was added activated charcoal (5 g/100 ml) to remove steroid, after which the mixture was stirred for 24 hours at 4°C , and centrifuged at 1650 g for 30 min. The supernatant was recentrifuged twice at 24,000 g for 60 min. to remove charcoal and cellular contaminants; then the fluid was filtered through $0.45\ \mu\text{m}$ (MILLEX-HA) and $0.22\ \mu\text{m}$ (MILLEX-GV) millipore filters. In all control groups sterile physiological saline was used. Before charcoal-treatment, the porcine FF contained $8.2 \pm 0.24\ \text{ng/ml}$ of estradiol-17 β and $143 \pm 5.2\ \text{ng/ml}$ of progesterone (mean \pm S.D.), respectively. After charcoal-treatment, estradiol-17 β level was $0.053 \pm 0.003\ \text{ng/ml}$ and progesterone level was $0.43 \pm 0.052\ \text{ng/ml}$. (Radioimmunoassay of these hormones was performed by The Fukuyamashi Clinical Assay Center.) Percentages of residual steroids were approximately 0.6% for estradiol-17 β and 0.3% for progesterone, comparable to values reported by other investigators^{2,3}).

Injection schedule and methods of observations

In treatment A in group 1, 5 ml of CTPFF were injected subcutaneously at 8-hour intervals for $4\frac{2}{3}$ days (15 times) in each doe. The does were restrained in a buck's hutch by hand to allow 3 matings⁴) immediately after the final injection of CTPFF without hCG administration. In other groups (treatment B in group 1, group 2 and group 3), 4 ml of CTPFF were subcutaneously injected at 12-hour intervals for $4\frac{1}{2}$ days (10 times) in each doe. The does in treatment B of group 1 were mated at the same manner as in treatment A of group 1, but the does in groups 2 and 3 were injected with 10 IU (group 2) or 15 IU (group 3) of hCG into the marginal vein of the ear simultaneously with the final injection of CTPFF. The does were laparotomized at 4 days (groups 1 and 2) or at 7 days (group 3) after the final injection of CTPFF; corpora lutea in ovulated does were counted and the diameters of visible ovarian follicles were measured ($> 1\ \text{mm}$) in non-ovulated does.

Appearance of vulva

The appearance of the vulva was inspected in all does once a day at midday from one day before injection of CTPFF to two days (group 1) or one day (groups 2 and 3) before laparotomy. The initial appearance of the vulva was scored as follows: a largely swollen, congested and purple or dark red vulva was designated as 3+; a somewhat swollen and red vulva was 2+; a mildly smaller and pink vulva was 1+; and a non-swollen, small and pale vulva was -. These designations were given the values 4, 3, 2 and 1, respectively, for statistical analysis.

Collection of blood and assay of FSH concentration in plasma

In group 1, only, blood samples were collected from the marginal ear vein for assay of FSH concentration at one day before injection of CTPFF; at one day, 3 days and 5 days (day of mating) of treatments, and at one and 2 days after mating (after end of treat-

ments) in both the A and B groups. Plasma FSH was measured by means of a double antibody radioimmunoassay utilizing the NIAMDD kit for rabbit FSH. This kit included standard rabbit FSH (AFP-538-C), iodinated rabbit FSH (AFP-538-C) and antiserum to rabbit FSH. Antibody against rabbit FSH (AFP-2-7-1) had been raised in guinea-pigs and was diluted to 1:100,000 for use in the assay. Goat-anti-guinea-pig- γ -globulin serum as the second antibody was diluted to 1:48 for use. Intra- and inter-assay coefficients were not calculated because of only one assay. The assay procedure accorded with the method by Scaramuzzi *et al.* (1972)⁵.

RESULTS

All does injected with CTPFF did not ovulate in both treatments A and B in group 1, even though they had copulated three times. However, the percent of ovulated does was 20 (2/10) in control A and 33 (2/6) in control B (Table 1). Many large blood follicles were found in ovaries of 6 non-ovulated does (4 does in control A, one doe in control B and another in treatment B). When the number of follicles – which were tentatively classified as 1.0 mm in diameter, 1.5-mm size, 2.0-mm size and larger than 2.5-mm size – was compared macroscopically, the number of follicles of larger size (1.5 and 2.0 mm) was comparatively smaller in non-ovulated ovaries of treatments A and B than in those of controls A and B, respectively (Table 2). The values for appearance of the vulva were significantly decreased from one day after the beginning of treatment in non-ovulated does of treatment A, and from 2 days after in those of treatment B, in comparison with controls (Figs. 1 and 2). FSH levels in all blood samples in CTPFF groups were undetectable during the treatment, showing levels below the minimum concentration detectable by the method used (< 0.60 ng/ml). However, in only 2 samples we were able to measure FSH levels in controls. Elevation in FSH levels was recognized in 4 does (2 in treatment A and 2 in treatment B) at 1–2 days after the end of treatments in the treated groups. Ovulated control does showed measurable concentrations at 1–2 days after matings (Table 3).

Table 1. Numbers of ovulated does and ovulations caused by matings or by administration of 10 IU or 15 IU of human chorionic gonadotropin (hCG) in does treated with successive injections of charcoal-treated porcine follicular fluid (CTPFF) or saline solution for about 5 days.

Group	Treatment	Method of induced ovulation	No. of ovulated does vs. No. of total does	No. of ovulations (mean \pm S.D.)	
1	A	CTPFF	mating	0/10	0
		saline	mating	2/10	9.5 \pm 3.5
	B	CTPFF	mating	0/5	0
		saline	mating	2/6	10.5 \pm 0.7
2	CTPFF	10 IU hCG injection	3/6	1.7 \pm 0.6**	
	saline	10 IU hCG injection	6/8	7.5 \pm 2.5	
3	CTPFF	15 IU hCG injection	6/6	3.0 \pm 2.2**	
	saline	15 IU hCG injection	7/7	10.9 \pm 1.2	

** p < 0.01

Table 2. Numbers of ovarian follicles (mean \pm S.D.) of different diameter in ovulated or non-ovulated does 96 hours (groups 1 and 2) or 168 hours (group 3) after successive injections of charcoal-treated porcine follicular fluid (CTPFF) or saline solution for about 5 days.

Group	Treatment	Ovulatory response ^{a)}	Follicular diameter (mm)				
			1.0	1.5	2.0	≥ 2.5	
1	A	CTPFF	no	6.5 \pm 3.6*	1.4 \pm 2.6	0.2 \pm 0.7**	0.2 \pm 0.9
		saline	no	3.9 \pm 3.2	3.2 \pm 3.8	2.3 \pm 2.0	0.5 \pm 1.2
	B	CTPFF	no	5.5 \pm 2.5	1.5 \pm 2.8	0	0
		saline	no	5.0 \pm 5.5	3.1 \pm 2.4	1.6 \pm 1.9	0
2	CTPFF	no	5.2 \pm 4.3	2.8 \pm 2.9	0.2 \pm 0.4	0	
		no	3.0 \pm 3.0	4.7 \pm 3.1	0.7 \pm 1.2	0	
	saline	ov	9.2 \pm 4.2	0	0	0	
		ov	8.0 \pm 4.9	1.8 \pm 1.8	0.3 \pm 0.7	0.2 \pm 0.4	
3	CTPFF	ov	9.8 \pm 8.5	5.3 \pm 4.1	0.3 \pm 0.9	0	
	saline	ov	9.8 \pm 5.2	4.8 \pm 3.0	1.2 \pm 1.9	0	

a) no, non-ovulated does; ov, ovulated does. * p < 0.05; ** p < 0.01.

Table 3. Mean concentrations of follicle-stimulating-hormone in peripheral blood (ng/ml) from one day before the successive injections of charcoal-treated porcine follicular fluid (CTPFF) or saline solution to two days after the end of treatments (group 1).

Group	Treatment	Days of sampling ^{f)}						
		before	period of injections				after	
		1	2	4	6	e)	7	8
A	CTPFF ^{a)}	1.15 (1/11 ^{g)}) c)	- d)	-	-	-	1.50 (1/11)	1.35 (1/11)
	saline a)	0.85 (1/10)	-	-	1.90 (2/10)	-	-	1.15 (3/10)
	ovulated does ^{b)}	-	-	-	-	-	2.63 (1/2)	1.10 (2/2)
B	CTPFF ^{a)}	-	-	-	-	-	0.60 (2/5)	-
	saline ^{a)}	-	-	-	-	-	-	-
	ovulated does ^{b)}	-	-	-	-	-	2.12 (2/2)	1.20 (2/2)

a) Samples obtained from non-ovulated does.

b) All ovulated does are in control.

c) Figures in parentheses show the number per total of samples.

d) Samples of this minus sign are undetectable in measurements.

e) Matings were performed at the end of treatments.

f) Figures of days are same in Figs. 1 and 2.

g) One of 11 does was dead before laparotomy.

In group 2, the percent of ovulated does was 50 (3/6) in the treated group and 75 (6/8) in the control group following administration of 10 IU of hCG. The number of ovulations was significantly lower in the treated group than in the control group (Table 1). Numbers of large follicles in both ovulated and non-ovulated does were small in the treated group in comparison with each control (Table 2). The values for appearance of

the vulva decreased from 1 day after CTPFF injection in ovulated does (Fig. 3) and from 2 days in non-ovulated does of the treated group (Fig. 4); then the value began to increase from 2 days after the final injection in both ovulated and non-ovulated does. However, values for non-ovulated does in the control decreased from 2 days after the final injection, in contrast.

In group 3, all does ovulated in both treated and control groups after administration of 15 IU of hCG, but the number of ovulations was significantly lower in the treated group than that in controls (Table 1). The number of follicles did not vary remarkably between treated and control groups in comparison with those in groups 1 and 2 (Table 2). The value for appearance of the vulva decreased significantly from 1 day after the beginning of treatment. However, the value began to increase from 3 days after the final CTPFF injection and no difference was noted between treated and control groups thereafter (Fig. 5).

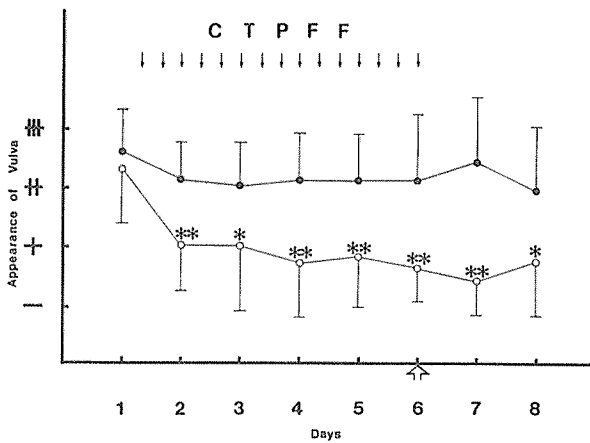


Fig. 1. Effects of successive administrations of charcoal-treated porcine follicular fluid (CTPFF) to 10 does (treatment A in group 1) on vulvar appearance (○, mean ± S.D.), in comparison with 8 does (●, mean ± S.D.) which were given physiological saline in the same manner but were not induced to ovulate by matings after the end of the successive injections. The evaluation of the appearance of the vulva is given in the text. †, dose of CTPFF or saline; ↗, mating; *p < 0.05, **p < 0.01.

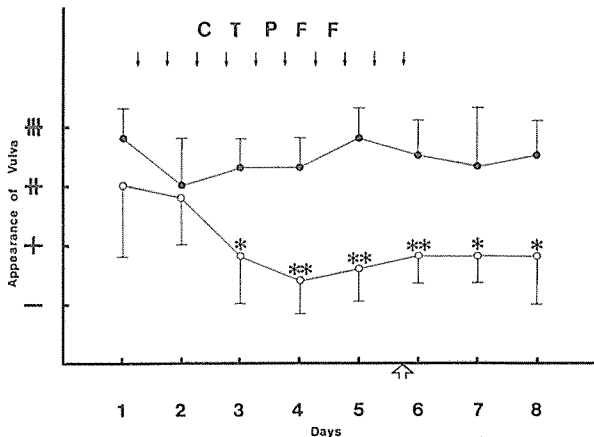


Fig. 2. Effects of successive administrations of charcoal-treated porcine follicular fluid (CTPFF) to 5 does (treatment B in group 1) on vulvar appearance (○, mean ± S.D.), in comparison with 4 does (●, mean ± S.D.) which showed no ovulations after being mated at the end of saline administrations in the same manner. The evaluation of the appearance of vulva is given in the text. †, dose of CTPFF or saline; ↗, mating; *p < 0.05, **p < 0.01.

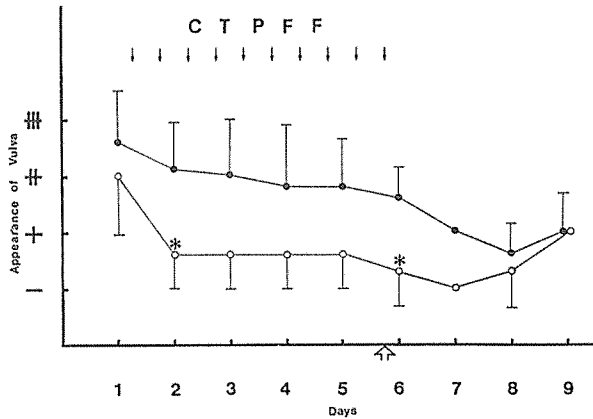


Fig. 3. Effects of successive administrations of charcoal-treated porcine follicular fluid (CTPFF) to 3 does in group 2 which were ovulated by an administration of 10 IU of hCG at the end of the treatments, on vulvar appearance (○, mean ± S.D.), in comparison with 6 does (●, mean ± S.D.) which were given saline in the same manner and ovulated by same procedure. The evaluation of the appearance of vulva is given in the text. ↓, dose of CTPFF or saline; ↗, injection of 10 IU of hCG; *p < 0.05.

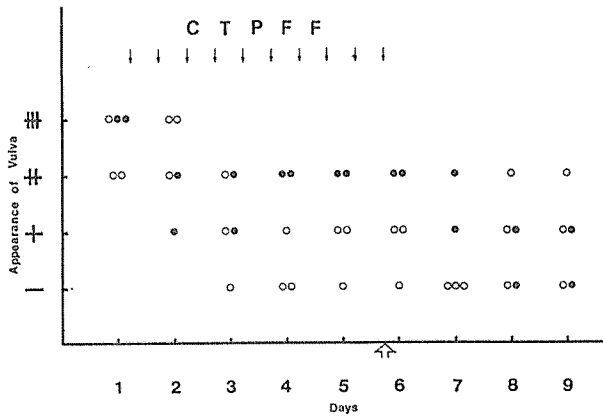


Fig. 4. Effects of successive administrations of charcoal-treated porcine follicular fluid (CTPFF) to 3 does in group 2, which did not ovulate following administration of 10 IU of hCG at the end of treatments, on vulvar appearance (○), in comparison with 2 does (●) which were given saline in the same manner and showed no ovulation by the same procedure. The evaluation of the appearance of vulva is given in the text. ↓, dose of CTPFF or saline; ↗, administration of 10 IU of hCG.

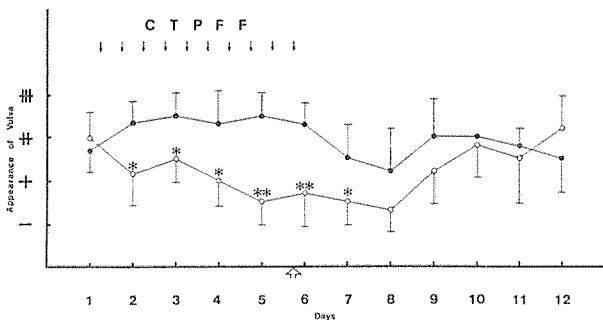


Fig. 5. Effects of successive administrations of charcoal-treated porcine follicular fluid (CTPFF) to 6 does in group 3, which were ovulated by administration of 15 IU of hCG at the end of treatments, on vulvar appearance (○, mean ± S.D.), in comparison with 7 does (●, mean ± S.D.) which were given saline in the same manner and ovulated by the same procedure. The evaluation of the appearance of vulva is given in the text. ↓, dose of CTPFF or saline; ↗, administration of 15 IU of hCG; *p < 0.05, **p < 0.01.

DISCUSSION

In group 1, 3 successive injections of CTPFF per day for about 5 days in treatment A and 2 injections in treatment B completely inhibited ovulation of mated does, although percentages of ovulated does were 20 (treatment A) and 33 (treatment B) in both controls. In group 2, a dosage of 10 IU of hCG gave rise to ovulation in 50% of treated does injected at 12-hour intervals for about 5 days, and in 75% of the control. This suggests that administration of 10 IU of hCG gives rise to a stronger stimulation of the

rabbit ovulatory mechanisms than does the copulatory stimulus.

The number of ovulations was significantly low in treated does of group 2 although the difference in fraction of does which ovulated between treated and control animals was not large. Furthermore, administration of 15 IU of hCG induced ovulations in all does in group 3 whether CTPFF was injected or not. The number of ovulations, however, was also significantly reduced in the treated group.

Existence of inhibin (or folliculostatin) in ovarian follicular fluid has been confirmed in various mammalian species, including rabbits, by reduction of FSH levels in blood of animals after administrations of steroid-free follicular fluid⁶⁻¹³). However, in the present estimations of FSH concentrations in rabbit plasma, many levels were too low to measure in both treated and control groups. The reason why FSH concentrations were undetectable in most samples of this experiment is not clear. KANEMATSU^{14,15}) reported that the postovulatory second surge of FSH in rabbits was detectable by the same assay method used in the present study, and that serum FSH levels before coitus were too low to measure. Individual values estimated after ovulation in his experiments were mostly lower than those reported by other investigators¹⁶⁻¹⁸), in spite of using the same kit for rabbit FSH.

In the present study, however, samples of all ovulated does showed relatively high levels of FSH one and/or two days after matings; this may be due to the second surge of FSH. In treated does which did not ovulate after mating, no samples showed detectable levels during the period of injection of CTPFF, though 3 samples at one day after treatment and 1 sample at 2 days after treatment did so. Perhaps those elevations in FSH concentration after treatment were caused by the rebound phenomenon following FF treatment, as suggested by DEPAOLO *et al.* (1979)⁹) and PHILLIPS *et al.* (1982)¹⁹) in rats, by MILLER *et al.* (1982)²⁰) in ewes, and by CHANNING *et al.* (1981)²) in monkeys. According to MILLS and COPLAND (1982)²¹), injections of porcine FF into rabbits completely blocked the second release of FSH (FSH-II) at 24 hours *post coitum* (*p.c.*) and delayed it until 60 hours *p.c.*; furthermore the FSH levels at 60 hours *p.c.* were nearly 3 times greater than those measured at 24 hours *p.c.* Then, the elevation of FSH after treatments in our experiments should be caused by a rebound in FSH release. But 3 samples in controls showed elevated FSH concentration after injections of saline solution in non-ovulated does. We cannot give any explanation of this phenomenon. Regardless of data too ambiguous to demonstrate the FSH-suppressive activity of CTPFF, the tendency of rebound in FSH levels after CTPFF treatments in the present study may suggest the existence of FSH-suppressive activity in CTPFF.

The only external sign of estrus in rabbits is still congestion and coloration of the vulva, although estrus is not sharply demarcated and vulvar appearance is not always a sign of desire to copulate²²). The estrous rabbit has follicles above a certain size²³) and the vulvar state may correlate with sexual activities^{24,25}). The present study showed that the value for appearance of the vulva decreased clearly within 1 or 2 days after the beginning of CTPFF treatments in all groups. The number of large follicles (1.5 and 2.0

mm in diameter) in non-ovulated does treated with CTPFF was considerably reduced in groups 1 and 2. This indicates that the injections of CTPFF suppressed follicular development and reduced the secretion of estrogen from follicles, and caused the fading of vulvar color. In addition, the state of appearance of the vulva recovered within 2 or 3 days after the end of treatments. The recovery may be due to the rebound phenomenon in FSH concentrations, as was stated previously.

In control groups of groups 2 and 3, the value for vulvar appearance decreased after dosage of hCG. The levels of estradiol in ovarian venous blood after ovulation remain very low to undetectable²⁶⁾, and this may lead to decrease of values of vulvar appearance in controls. However, the value for non-ovulated control does in group 2 tended to decrease after saline treatment, also. The reason is not clear.

At 7 days after treatments, the numbers of follicles of different size-categories did not vary between treated and control groups. This suggests that undeveloped follicles due to CTPFF became developed follicles due to a rebound in FSH concentration, resulting in elevation of the values for appearance of the vulva after treatments.

SUMMARY

The effect of successive administrations of charcoal-treated porcine follicular fluid (CTPFF) on ovulation in rabbits was examined.

In group 1, fifteen does mated after receiving 15 injections of 5 ml of CTPFF at 8-hour intervals (treatment A) or 10 injections of 4 ml at 12-hour intervals (treatment B) showed completely suppressed ovulation. Control does which were treated with saline solution ovulated (20% of does in control A and 33% in control B). Non-ovulated does treated with CTPFF showed a decrease in large follicles (≥ 1.5 mm size in diameter) 4 days *post coitum*. Vulvar swelling and coloration receded 1 day (treatment A) and 2 days (treatment B) after the beginning of CTPFF treatments. Follicle-stimulating-hormone (FSH) concentrations in peripheral blood were examined by radioimmunoassay, but no samples had detectable levels during the treated period in CTPFF groups. But elevation in FSH levels was noted in 4 does in treated groups after treatments.

In group 2, 6 does which had received 10 injections of 4 ml of CTPFF at 12-hour intervals were given 10 IU of hCG, resulting in ovulations in 50%. The same dose of hCG caused ovulation in 75% of control animals (8 does). The number of ovulations was significantly lower in the CTPFF groups than in controls ($p < 0.01$). Numbers of large follicles were fewer in ovaries of treated non-ovulated does than that in controls 4 days *post coitum*. Vulvas became smaller and more faded from treatment with CTPFF, also.

In group 3, 15 IU of hCG was used for ovulation induction in 6 does which were treated with CTPFF in the same manner as group 2. This gave rise to ovulation in all does in both treated and control (7 does) groups. However, the number of ovulations was significantly lower in the CTPFF group than in the controls ($p < 0.01$). The appearance of the vulva showed a trend similar to that in groups 1 and 2. But the number of large follicles in ovulated does did not vary at 7 days after the end of CTPFF

treatments between treated and control groups. This indicates that follicular development which is inhibited by CTPFF treatments may be resumed by a rebound phenomenon in FSH levels.

It is concluded that successive injections of CTPFF for several days affected ovulation in our rabbits, not only reducing the number of does which ovulated, but also the number of ovulations in does which were caused to ovulate by administration of hCG.

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ステロイド除去豚卵胞液の投与が家兔の 排卵に及ぼす抑制効果について

吉田康則・前田照夫・寺田隆登・堤 義雄・平田武彦・兼松重任

活性炭で処理した豚卵胞液 (CTPPF) を家兔に投与し、交尾刺激又は hCG 投与により誘起される排卵について検討した。

1. CTPPF を 5 ml ずつ 8 時間おきに 15 回連続投与 (10 羽)、又は 4 ml ずつ 12 時間おきに 10 回投与 (5 羽) し、最終投与時に強制的に交尾させたが、処理区は全く排卵せず、対照の生理的食塩水投与区で 16 羽中 4 羽が排卵した。交尾後 4 日目の処理区家兔卵巣では大型卵胞数が有意に減少しており、外陰部の腫脹・潮紅の度合も CTPPF 投与開始後 1～2 日で低下した。末梢血中 FSH 量の測定結果については (RIA 法)、CTPPF 処理期間中処理区の全てのサンプルが測定限界値以下であったが、投与終了後 1～2 日に高い値を示したものがあり、CTPPF 処理に対するリバウンド現象が起きている可能性が考えられた。
2. 4 ml の CTPPF を 12 時間おきに連続 10 回投与し、hCG 10 IU を静注したところ、6 羽中 3 羽が排卵し、対照区では 8 羽中 6 羽が排卵した。排卵数は処理区で有意に減少していた。非排卵個体の大型卵胞数は処理区で減少していた (hCG 投与後 4 日)。外陰部の状態も投与開始後衰退した。
3. 前項 2 と同様に処理し、hCG を 15 IU 投与した。処理区、対照区とも全個体排卵したが、排卵数は処理区で有意に少なかった。hCG 投与後 7 日目の大型卵胞数には両区間で有意差はなかった。外陰部の状態は矢張り CTPPF 投与開始後 2 日目から衰退したが、投与終了後 4 日目で降回復して両区間に差は認められなくなった。

以上の結果、数日間の CTPPF 連続投与が家兔の排卵に対して抑制的効果を示し、排卵数をも減少させることが知られた。