Subnormal Function of Rabbit Corpus Luteum Caused by the Administration during Follicular Phase of Steroid-Free Porcine Follicular Fluid

Yasunori YOSHIDA*, Shinobu NAGAHAMA, Yūko ITO, Teruo MAEDA, Takato TERADA, Yoshio TSUTSUMI, Kiyoshi OKUDA*, Nobue SUMI*, and Kunitada SATO*

Animal Reproduction Laboratory, Faculty of Applied Biological Science, Hiroshima University, Fukuyama-shi 720
*Department of Veterinary Obstetrics and Gynecology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro-shi 080

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Abstract Effects of injections of charcoal-treated porcine follicular fluid (CTPFF) during 5 days in the follicular phase on functions of the corpus luteum in pseudopregnant rabbits were examined. Three ovulatory does which were ovulated by administration of 10 IU of hCG after ten 4-ml injections of CTPFF at 12-hour intervals showed no difference from controls in wet weights of either their ovaries or corpora lutea on day 4 of pseudopregnancy, even though there was a significant difference in the number of ovulations in comparison with 6 control does (group 1). The number of large follicles (≥1.5 mm diameter) in treated does was less than that in the control. Progesterone (P) levels in peripheral plasma were lower in treated does on day 4 of pseudopregnancy than in control does, in spite of higher levels during the period of CTPFF treatments. In 6 does which were ovulated by 15 IU of hCG after injections of CTPFF in the same manner as in group 1, wet weights both of ovaries and corpora lutea on day 7 decreased, and number of ovulations was very few in comparison with 7 control does (group 2). Numbers of follicles of various sizes were not different between treated and control groups. Progesterone levels in treated dose remained at lower levels from 3 days to 7 days after ovulation than in controls. In treated does, luteal cells were polygonal and there were some regressive tissue changes. Ten days after administration of 15 IU (treatment A in group 3) or 30 IU (treatment B in group 3) of hCG following CTPFF treatments, all does (7 does for treatment A, 5 does for treatment B, 4 does for control) showed similar mean ovarian weights. However, the mean weight of corpora lutea in does of treatment B was significantly less than that in does of treatment A. Mean number of ovulations in treatment B was the same as in the control, but that in treatment A was significantly smaller. Numbers of follicles in the 1.0-1.5 mm sizes in treatment B in group 3 were greater than in treatment A and the control. Progesterone levels in treated does remained at lower levels than in controls from 3 to 10 days after ovulation. Total wet weights of corpora lutea were highly correlated (p<0.01) with P levels in all groups. These results indicate that

a) Present address: Takarazuka Research Center, Sumitomo Chemical Co., Ltd., Takarazuka-shi 665

successive injections of CTPFF for several days during the follicular phase give rise to subnormal function in the corpus luteum on day 7 of pseudopregnancy in rabbits.


**Key words:** follicular fluid, inhibin, rabbit reproduction, corpus luteum, plasma progesterone

It is well established that porcine follicular fluid (PFF) possesses inhibin-like activity which selectively suppresses serum follicle-stimulating hormone (FSH) levels in several animal models, including rabbits1-4. We recently reported that subcutaneous injections of charcoal-treated porcine follicular fluid (CTPFF) successively for 5 days completely suppressed ovulation in mated rabbits, and reduced the number of ovulations in does treated with human chorionic gonadotropin (hCG)5,6. Mildly aplastic corpora lutea were found macroscopically on day 7 of pseudopregnancy following treatments with CTPFF for 10 days in this species6. STOUFFER and HODGEN7 reported that administration of CTPFF to rhesus monkeys at the onset of the menstrual cycle impaired the subsequent function of the corpus luteum. The luteal dysfunction in CTPFF-treated monkeys was documented by subnormal serum progesterone levels, lower wet weight of the corpus luteum, and lower activity of steroidogenesis in dispersed luteal cells _in vitro_. Subsequently, STOUFFER _et al._8 demonstrated that CTPFF treatments given to rhesus monkeys in the early follicular phase vitiated development of the dominant follicle and the related corpus luteum; but similar treatments at midluteal phase did not suppress concurrent luteal function or subsequent folliculogenesis. According to EL-SHEIKH and NALBANDOV9 administration of PFF preparation for 5 consecutive days, beginning on the day of mating, in rabbits gave rise to significantly decreased number of implantation sites and of living fetuses by day 16 of gestation, and caused significantly lower concentrations of progesterone and 20α-hydroxy-4-pregnen-3-one in both ovarian effluent blood and corpus luteum on day 16. Therefore, injections of CTPFF may affect the function of corpus luteum in rabbits. In the present study, effects of injections of CTPFF during the follicular phase on luteal function of pseudopregnant rabbits were examined.

**Materials and Methods**

*Animals* Fifty virgin, female, adult Japanese White rabbits, weighing 3.0-4.0 kg, were used. 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 — 5 for treatment and 8 for control in group 1; 8 for treatment and 9 for control in group 2; 9 for treatment A, 5 for treatment B and 6 for control in group 3. Two dose in treatment and 2 control animals of group 2 were supplied only for histology. One control doe in each of groups 1 and 2 was used only for measurement of wet weight of ovaries and corpora lutea, avoiding blood collection, and 2 dose each in treatment and control of groups 1 and 3 (except treatment B) were used only for blood collection.
Preparation of charcoal-treated porcine follicular fluid (CTPFF) Porcine ovaries were collected at a local meat-packing plant, immediately placed on ice and transported to our laboratory (Hiroshima University, Fukuyama). Follicular fluid was aspirated mainly from small and medium follicles, excluding ovaries having corpora lutea or cystic follicles. The fluid was pooled in a plastic bottle and stored in a freezer at \(-20^\circ\)C until use. The frozen porcine follicular fluid (PFF) was slowly melted in a refrigerator at 4\(^\circ\)C. The melted PFF was added to activated charcoal (5g/100ml) to remove steroid, stirred for 24 hours at 4\(^\circ\)C, and centrifuged at 1,650g for 30 min. The supernatant was recentrifuged twice at 24,000g for 60 min to remove charcoal and cellular contaminants completely. Then the fluid was filtered twice through 0.45-µm (MILLEX-HA) and 0.22-µm (MILLEX-GV) millipore filters.

Injection schedule and methods of observations In all treatment groups, 4 ml of CTPFF were injected subcutaneously at 12-hour intervals for 5 days (10 times) in each doe. In all control groups, sterile physiological saline was used for injections. To induce pseudopregnancy the does were administered 10 (group 1), 15 (group 2 and treatment A in group 3) or 30 (treatment B in group 3) IU of hCG simultaneously with the final injection of CTPFF. The day of hCG administration was designated as day 0 of pseudopregnancy. On day 4 (group 1), 7 (group 2) or 10 (treatments A and B in group 3) of pseudopregnancy, the does were laparotomized to count the number of corpora lutea and to measure the diameter of visible ovarian follicles (≥1.0 mm). Thereafter, the does were euthanized and their ovaries were removed and weighed. All corpora lutea from most does were enucleated from ovaries to measure their weights. Ovaries from 4 does in group 2 were fixed in Bouin’s solution, embedded in paraffin, sectioned serially at 7 µm, and stained with hematoxylin and eosin to observe luteal cells microscopically.

Sampling of blood and assay of progesterone in plasma In all groups, blood samples were collected from the marginal ear vein for assay of progesterone (P) one day before and 0, 2 and 4 (4 hours after hCG injection) days after the first injection of CTPFF, and 1 day after hCG administration. Additional samples were collected at 2 and 4, 3, 5 and 7, and 3, 5, 7 and 10 days after hCG injection from the does in groups 1, 2 and 3, respectively.

Progesterone concentrations in peripheral plasma were determined by a radioimmunoassay described by MAKINO\(^{10}\). Peripheral plasma (0.01–0.2 ml) was extracted with ethylether. The range of the water blank was 0 to 0.013 ng/ml, the recovery rate was 90.0 ± 10.4 percent (mean ± S. D.), and a straight line was obtained in the range between 0 and 1 ng as the standard curve. Means of within-assay variation and between-assay variation were 0.4270 ng/ml (C. V., 8.5%) and 0.5067 ng/ml (C. V., 4.0 %), respectively.

Statistical analysis All data on wet weight of ovaries and corpora lutea, number of follicles, and P levels in all groups and correlation of weights of corpora lutea with P levels were analyzed using STUDENT’s t test.
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Results

Wet weights of ovaries and corpora lutea

Wet weights of ovaries and corpora lutea in all groups are shown in Table 1. Although weights of ovaries on 4 and 10 days of pseudopregnancy did not differ between treated and control groups, those of treated group in group 2 were significantly decreased. No differences were noted in weights of corpora lutea between treatment and control in group 1 and between treatment A and control in group 3. However, the significant difference was observed between treatment and control in group 2. As CTPFF treatments reduced the number of ovulations significantly, 5 does in treatment B in group 3 were injected with 30 IU of hCG, resulting in no significant difference from the control in the number of ovulations.

Table 1. Mean wet weights of ovaries and corpora lutea 4 (group 1), 7 (group 2) and 10 (group 3) days of pseudopregnancy following injections of charcoal-treated porcine follicular fluid (CTPFF) or saline solution (S) for 5 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of does</th>
<th>No. of corpora lutea per doe (mean±S.D.)</th>
<th>Wet weight(mean±S.D., mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ovary</td>
<td>Corpus luteum</td>
</tr>
<tr>
<td>1</td>
<td>CTPFF+10 IU hCG</td>
<td>3</td>
<td>1.7±0.5**</td>
<td>379.5±66.6</td>
</tr>
<tr>
<td></td>
<td>S+10 IU hCG</td>
<td>6</td>
<td>7.5±2.5</td>
<td>395.3±83.0</td>
</tr>
<tr>
<td>2</td>
<td>CTPFF+15 IU hCG</td>
<td>6</td>
<td>3.0±2.2**</td>
<td>261.7±108.0**</td>
</tr>
<tr>
<td></td>
<td>S+15 IU hCG</td>
<td>7</td>
<td>10.9±1.2</td>
<td>423.2±158.4</td>
</tr>
<tr>
<td>3</td>
<td>(A) CTPFF+15 IU hCG</td>
<td>7</td>
<td>1.4±0.53**</td>
<td>419.1±208.7</td>
</tr>
<tr>
<td></td>
<td>(B) CTPFF+30 IU hCG</td>
<td>5</td>
<td>7.8±3.3</td>
<td>528.8±222.0</td>
</tr>
<tr>
<td></td>
<td>S+15 IU hCG</td>
<td>4</td>
<td>10.0±3.6</td>
<td>426.5±116.5</td>
</tr>
</tbody>
</table>

Significantly different compared with control at *P<0.05 and **P<0.01.

Table 2. The numbers of ovarian follicles (mean±S.D.) of different diameter in each ovary 4 (group 1), 7 (group 2) and 10 (group 3) days of pseudopregnancy following injections of charcoal-treated porcine follicular fluid (CTPFF) of saline solution (S) for 5 days and induction of ovulation with 10, 15 or 30 IU of hCG

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Follicular diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>CTPFF+10 IU hCG</td>
<td>9.2±4.2</td>
</tr>
<tr>
<td></td>
<td>S+10 IU hCG</td>
<td>8.0±4.9</td>
</tr>
<tr>
<td>2</td>
<td>CTPFF+15 IU hCG</td>
<td>9.8±8.6</td>
</tr>
<tr>
<td></td>
<td>S+15 IU hCG</td>
<td>9.8±5.2</td>
</tr>
<tr>
<td>3</td>
<td>(A) CTPFF+15 IU hCG</td>
<td>7.6±4.6</td>
</tr>
<tr>
<td></td>
<td>(B) CTPFF+30 IU hCG</td>
<td>12.5±3.9*</td>
</tr>
<tr>
<td></td>
<td>S+15 IU hCG</td>
<td>7.4±3.5</td>
</tr>
</tbody>
</table>

*Significantly different compared with control at p<0.05.
ovulations. Although mean weights of ovaries of all subgroups in group 3 did not vary significantly, weights of corpora lutea in treatment B in group 3 were significantly less than those in the control. Mean total weights of corpora lutea per doe were remarkably decreased in all CTPFF-treated groups in comparison with each control, except in treatment B in group 3.

**Follicular sizes** The number of large follicles (≥1.5 mm in diameter) in group 1 appeared to be decreased by CTPFF, but the difference in the number of large follicles between treated and control groups was smaller in group 2 and treatment A in group 3. In treatment B in group 3, however, the number of follicles of 2.0-mm size was smaller than in treatment A in group 3 and the control, though the numbers of follicles of 1.0-mm and 1.5-mm sizes were greater than in treatment A in group 3 and the control (Table 2).

**Levels of progesterone in peripheral plasma** There were no differences between treated and control groups in P levels before and during periods of CTPFF treatments in group 2 (Fig. 2) and 3 (Fig. 3). In all groups, P levels were elevated markedly at 4 hours after hCG injection and declined to the follicular levels the next day, followed by

![Fig. 1. Progesterone levels (mean±S.D.) in peripheral plasma in group 1. Five does were administered 4 ml of charcoal-treated porcine follicular fluid (CTPFF) 10 times at 12-hour interval and induced to ovulate by 10 IU of hCG at the final injection of CTPFF (), in comparison with 7 does given saline solution (S) in the same manner and induced to ovulate likewise (○). ↓, dose of CTPFF or S; †, administration of hCG.

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increasing levels to 4 (group 1), 7 (group 2) and 10 (group 3) days after hCG administration. Comparing the rates of increase in P levels after the ovulation treatment, lower P levels were evident in all treated groups: that is, on day 4 of pseudopregnancy in group 1, and after day 3 in groups 2 and 3. In spite of the lack of difference in mean numbers of corpora lutea between treatment B in group 3 and the control in group 3 (Table 1), the rate of increase in curve of mean P levels in treatment B in group 3 after ovulation was intermediate between treatment A in group 3 and control (Fig. 3).

**Correlation between weights of corpora lutea and progesterone levels**  Total wet weights of corpora lutea in each doe were highly correlated (P<0.01) with P levels in peripheral plasma at laparotomy in all groups (Fig. 4).

**Microscopic observations of corpora lutea**  Most luteal cells on day 7 of pseudopregnancy (group 2) were polygonal in shape in treated does (Fig. 5), in contrast to somewhat elongated, ovoid luteal cells in control does (Fig. 6). It was noted that clumps of fibroblast-like cells appeared frequently among luteal cells in treated does. The luteal cells in control does were linked closely each other, forming strips which were arranged radially around the central cavity of the corpus luteum. However, no

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**Fig. 2.** Progesterone levels (mean±S.D.) in peripheral plasma in group 2. Eight does were administered 4 ml of charcoal-treated porcine follicular fluid (CTPFF) 10 times at 12-hour interval and induced to ovulate by 15 IU of hCG at the final injection of CTPFF (○), in comparison with 8 does given saline solution (S) in the same manner and induced to ovulate likewise (●). ↓, dose of CTPFF or S; ↑, administration of hCG; *p<0.05; **p<0.01.
Fig. 3. Progesterone levels (mean±S.D.) in peripheral plasma in group 3. Nine does (treatment A) and 5 does (treatment B) were administered 4ml of charcoal-treated porcine follicular fluid (CTPFF) 10 times at 12-hour interval and induced to ovulate by 15 IU (treatment A, ◦) or 30 IU (treatment B, △) of hCG, in comparison with 6 does given saline solution (S) in the same manner and induced to ovulate by 15 IU of hCG (●). ↓, dose of CTPFF or S; ↑, administration of hCG; *p<0.05 or **p<0.01.

Fig. 4. Correlation between progesterone levels in peripheral plasma and the total wet weights of corpora lutea per doe in all groups. Figures (1, 2 and 3) show group numbers and T and C mean treatment and control, respectively.
Figs. 5 and 6. Photomicrographs of luteal cells on day 7 of pseudopregnancy in does which received CTPFF (Fig. 5) or saline (Fig. 6) in group 2. †, clumps of fibroblastlike cells in Fig. 5; ‡, strips of luteal cells in Fig. 6. (× 120)

such a trend in arrangement of luteal cells in treated does was observed, and the density of luteal cells seemed to be lower in the treated does.

Discussion

Wet weights of individual corpus luteum and P levels in peripheral or ovarian venous blood, or in luteal tissue, have been investigated as reflecting functional activity of the corpus luteum by many investigators\(^{11,12}\). NASS et al.\(^{13}\) reported that the depressed FSH secretion throughout the follicular phase of the menstrual cycle in rhesus monkeys, particularly early in this phase, led to inadequate stimulation and
maturation of the preovulatory follicle and reduction of estradiol-17β (E2) secretion, and that ovulation of small or retarded follicles under those conditions gave rise to a deficient corpus luteum (both structurally and physiologically) and short luteal phase. To give direct evidence to support this finding, STOUFFER et al.8) administered CTPFF to rhesus monkeys on days 1–3 of the menstrual cycle to suppress serum FSH. The treatment induced subnormal serum E2 and P levels and lighter wet weights of corpus luteum at the midluteal phase. They14) also reported that luteal cells at the midluteal phase in CTPFF treated monkeys during days 3–5 after menses secreted significantly less P than in controls, and that the luteal cells from treated monkeys failed to respond to hCG with enhanced P production in vitro. In the present study, injections of CTPFF in rabbits during the follicular phase (before hCG injection) for 5 days resulted in low luteal function, as indicated by weights of individual corpora lutea and a somewhat degenerative picture in luteal tissue on day 7 of pseudopregnancy, and by peripheral P levels from day 3 on.

The number of ovulations induced by 10 or 15 IU of hCG was reduced significantly in CTPFF–treated does, and this confirmed our previous results5,6). It seems that the lower P levels in CTPFF–treated animals are due to a smaller number of corpora lutea and probably fewer total luteal cells in treated does than in the control animals. Evidence was obtained in the present study that plasma P levels were highly correlated with total weights of corpora lutea in each doe. If the weight of corpora lutea depends on the number of luteal cells, low P levels in treated groups could be due to fewer luteal cells. There is a possibility that the lower P levels in treatment B in group 3 are due to the smaller number of luteal cells in light–weight corpora lutea of the does in that group on day 10 of pseudopregnancy, because the mean number of corpora lutea was not significantly different from that in the control. In treatment A in group 3, P levels remained the lowest in spite of somewhat heavier corpora lutea. This may be due to the markedly decreased number of corpora lutea in treatment A in group 3, which led to reduction in the total weight of corpora lutea and in total numbers of luteal cell in this group.

The present study demonstrated clearly that the administration of CTPFF reduced the number of large follicles in ovulated does on day 4 of pseudopregnancy, as compared to control does. Recent reports15,16) clarified that the rabbit corpus luteum can develop and secrete P for 6 days from the beginning of pseudopregnancy in the absence of ovarian follicles which could supply estrogen as essential luteotropin in rabbits. LH or other pituitary hormones were not required for normal development, maintenance and regression of the corpora lutea. After day 6 of pseudopregnancy, the corpus luteum changed to estrogen–dependent tissue. Thus the significantly smaller weights and somewhat degenerative appearance in individual corpora lutea in treated does on day 7 in our study might be due to insufficient estrogen secretion from undeveloped follicles affected by CTPFF administrations, since the weights of corpora lutea on day 4 were not different between treated and control groups. But the number of large follicles was not different macroscopically on day 7 of pseudopregnancy between
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treated and control groups, regardless of a decreased number of them on day 4. This rapid recovery of development of follicles may be due to a rebound in FSH levels after the end of CTPFF injections, as suggested by other investigators\(^1\). The mean wet weight of corpora lutea in treatment A in group 3 was greater than in the control on day 10. It may be that a rebound in FSH levels stimulated the follicles, resulting in enhanced estrogen secretion which caused further development of corpora lutea. This speculation is based on the acutely increased number of follicles of 2.0-mm diameter as days passed after cessation of treatments (except for treatment B in group 3). The low ratio of the number of corpora lutea to the number of large follicles may also act additionally to develop corpus luteum in consideration of markedly smaller weight of corpora lutea in treatment B in group 3 in comparison with treatment A in group 3. The enhanced levels of P on the day of hCG administration were consistent with the results of other investigators\(^1\).

It is a general conception that two kinds of pituitary hormones, FSH and LH, and ovarian steroid hormones are required for continuous follicular development. Granulosa cells are the target for FSH, but not thecal cells; and granulosa cells and thecal cells give rise to luteal cells\(^9\). On day 7 of pseudopregnancy (group 2), the shape of luteal cells was polygonal predominantly, although the cells of control specimens were elongated-ovoid. The cellular size was apparently various in both specimens, and no clear difference in cellular size was recognized between them. However, clumps of fibroblast-like cells appeared frequently between luteal cells only in treated does. This may suggest that the corpus luteum on day 7 of pseudopregnancy in CTPFF-treated does was degenerative, and this means that CTPFF treatments were detrimental to development of the corpus luteum. According to STORMSHAK and CASIDA\(^2\), the existence of intercellular fibroblasts in the corpus luteum signals degenerative changes in the luteal tissue. A difference in the arrangement of luteal cells was marked at this stage. Strips which were densely filled with luteal cells were arranged radially around the central cavity of the corpus luteum in control does, while no such trend was found in treated does. The cellular configuration in the control gives an impression that the corpus luteum tissue was very active in the developmental process, in comparison with the deficient situation in treated animals. This is also suggested by the lower P levels in treated does, as mentioned in the present study.

CHANNING et al.\(^2\) stated that the injection of CTPFF in rhesus monkeys prevented follicular development and reduced the number of granulosa cells recovered from these follicles. CHARI et al.\(^2\) reported, in a morphological study, that degeneration of the granulosa cells following injection of highly purified inhibin was observed in rats. According to SOBOTTA\(^3\), the corpus luteum of the rabbit is derived from simple enlargement of granulosa cells, unaccompanied by division. From these facts, the decreased number of granulosa cells may result in decreased number of luteal cells. Then, in the present study, decreased wet weights of corpora lutea and subnormal features of luteal cells on day 7, and low levels of P from day 3 of pseudopregnancy following CTPFF treatments, must be due to a subnormal number of granulosa cells.
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during the follicular phase, affected by the injections of CTPFF.

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References

ステロイド除去豚卵胞液の排卵前投与による家児黄体機能の低下について

吉田康則・永瀬 祐・伊藤祐子・前田照夫・寺田隆登
堤 誠雄・奥田 潔*・住 延栄*・佐藤邦志*

活性炭処理豚卵胞液（CTPFF）4 ml を成熟家兔に12 時間おきに10 回皮下注射し（処理），最終投与時
に 10 IU の hCG を静注して排卵を誘起した。偽妊娠
4 日目の黄体数は処理区（3 羽）で对照区（生理食塩
水投与，6 羽）より有意に減少したが，卵巣と黄体の平
均重量には差はなく，大型卵胞数は処理区で減少してい
た。末梢血漿中のプロゲステロン（P）濃度は処理中対
照に比しやや高レベルを示したが，排卵時の上昇，その
後の急減は両区とも同様であり，偽妊娠4 日目のP レ
ベルは対照区の方が上昇していた。

処理後 15 IU の hCG 投与で排卵した6 羽では，黄
体数は対照区（7 羽）より有意に少なく，偽妊娠3 日目
の卵巣と黄体の平均重量も有意に減少した。卵胞の大き
さ別個体数には差はなかった。P 濃度では処理中および
排卵翌日まで両区間に差はなかったが，偽妊娠3 日目か
ら7 日目まで処理区は低い値を維持した。7 日目の黄体
組織には処理区で退行的微候が認められた。

処理後 15 IU（A 区，7 羽）または 30 IU（B 区，5
羽）の hCG を投与し，偽妊娠10 日目に検査したもの
では，卵巣重量も黄体重量も両区間に差はなかった。し
かし卵胞数ではB 区で1.0 〜1.5 mm 大のものが非常
に多く，2.0 mm 大のものが少なかった。P 濃度は偽
妊娠3 日から10 日まで処理区で低値を示し，特に A
区で著しかった。全体を通じ1 羽当りの総黄体重量と
P 濃度間には高い相関が認められた。

以上の結果より，卵胞期の家児に CTPFF を連続投
与すると，hCG による排卵誘起後の黄体形成は抑制さ
れるとともに排卵後7 日の黄体機能が低下することが示
唆された。

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