Prolonged Inhibitory Effect of Progesterone on Ovulation in the Domestic Hen (*Gallus domesticus*)

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Progesterone (P_4) as a positive feedback signal plays a pivotal role in the regulation of LH release occurring 4 to 6 hr before ovulation in the laying hen. Injection of LH induces P_4 , and P_4 injection induces LH release causing ovulation when those injections are made 8–10 hr before preovulatory LH release^{1–3}). P_4 may exert its stimulatory effect on the hypothalamus as well as other regions of the brain^{4–7}).

Recently, negative feedback of steroid hormones in the regulation of LH release has been suggested⁸). When P_4 is injected shortly after ovulation, the next ovulation is delayed²). In some, the ovary atrophies⁹). The present study was conducted to investigate the inhibitory effect of chronic administration of P_4 on the regulatory mechanisms of preovulatory LH release and to examine at which level of the hypothalamo–anterior pituitary–ovarian axis P_4 exerts the effect.

Material and Methods

Animals

Ninety crossbred laying hens (Goto 360, Goto Hatchery Inc. Japan), 10–14 months old and weighing 1.55–2.21 (averaging 2.01) kg, were maintained in individual cages. The lighting regimen was 14 hr light (0600–2000)/24 hr and food and water were always available. The recording system for the time of oviposition was the same as previously reported¹⁰). The experimental hens laid relatively regular sequences of 5 to 8 eggs on consecutive days in a sequence and each sequence was separated by a pause day. Ovulation was considered to occur 30 min after oviposition of the previous ovum in the series¹¹).

Design of Experiments

The present study consists of 4 experiments as summarized in Fig. 1. Before the initiation of the study, 5 cm sections of Silastic tubing (i.d. 2.64 mm, Corning Co. NY.) filled with P_4 crystal powder (Sigma Co. St. Louis) was implanted under the abdominal skin of hens. Two days after the implantation the Silastic tubing was removed and stored at -20°C . Hens used for pretreating of the Silastic tubes were not used in any of the 4 experiments. The time of ovulation was determined by the position of the ovum in the oviduct at autopsy (Fig. 1).

Experiment 1

Two Silastic tubings filled with progesterone (Pt) were implanted under the abdominal skin of each hens 1.5 hr after the second (C_2) ovulation of the sequence (24 hr before C_3 ovulation). Seven and 18 hens were killed by overdose of pentobarbital sodium at 45 hr and

Fig. 1. Schematic illustration of the experimental protocols. Progesterone filled in the Silastic tubing (Pt) was implanted s. c. 1.5 hr after the second ovulation (C₂) of a sequence. C₃, C₄ and C₅ designate subsequent, expected ovulations of third, fourth and fifth eggs of the sequence, respectively. Numbers of h indicate time (hr) after Pt implantation. Treatments were shown above and below each panel, respectively, for Experiments 2, 3 and 4.

73 hr after Pt implantation, respectively.

Experiment 2

One hundred micrograms of ovine LH (NIH-LH-S24) in 0.5 ml NaCl (9 g/l) solution was given intraperitoneally¹²⁾ 45 hr after Pt implantation in 13 hens as described in Experiment 1. Another 13 hens were injected 13.5 hr after C₂ ovulation (12 hr before C₃ ovulation) with LH but received no Pt treatment. The hens with Pt were killed 8 hr after injection but hens without Pt were killed 12 hr after the injection.

Experiment 2

Ten hens were injected intramuscularly with [Arg⁸]-LHRH (25 µg/hen, Peptide Institute Protein Research Foundation) in 0.5 ml NaCl solution 45 hr after Pt treatment as described in Experiments 1 and 2. Another 10 hens without Pt were given only LHRH 13.5 hr after C₂ ovulation.

Experiment 3

Eleven hens were injected with Progesterone (1.0 mg/hen, Oophormin Luteum, Teikoku-zoki Pharmaceutical Co.) into the breast muscle 45 hr after implantation. Eight hens without pretreatment were injected P₄ at 13.5 hr after C₂ ovulation.

Plasma Collection and Radioimmunoassay of Plasma Steroids and LH

The brachial vein was catheterized and in each hen blood samples were collected serially at 0, 2, 4, 6 and 45 hr after Pt implantation in the Experiment 1. In the Experiment 2, 3 and 4 blood was collected 0, 1, 2, 3 and 4 hr after each injection of LH, LHRH or P₄ in all hens whether or not they were pretreated with Pt. Usually 1.0–2.0 ml blood was collected for each sample. Plasma samples were stored at –20°C until use.

The concentrations of P_4 , testosterone (T) and estradiol-17 β (E_2) in plasma were measured by antiserum against each steroid (Teikoku Zoki Pharmaceutical Co.) using radioimmunoassays^{13,14}. The minimum detectable level was 25 pg/ml for P_4 , T and E_2 . The intra- and inter-assay coefficients of variation were 7.6 and 15.5% for P_4 , 6.5 and 13.8% for T, and 7.4 and 19.0% for E_2 , respectively.

Plasma concentrations of LH were measured one assay in duplicate using radioimmunoassay described by Hattori and Wakabayashi¹⁵. Rabbit anti-LH serum (Anti-AL-MH-# 1) and avian LH preparation (IEF-1) for radioiodination and standards were provided by Dr. K. WAKABAYASHI and Dr. M. HATTORI, Gunma University. The minimum detectable level of LH was 0.1 ng/ml and intraassay coefficient variation (CV) was 10.3%.

Statistical Analysis

Hormone data between Pt-treated and non-treated hens were compared by two-way analysis of variance (ANOVA). The mean values at different time within the same group were compared by Duncan's new multiple range test after analysis of variance (ANOVA). The rates of induced ovulation between groups and induction time for ovulation between groups within each experiment were compared by χ^2 -test and by Student's *t*-test, respectively.

Results

Effects of Pt Implantation on Spontaneous Ovulation and Plasma Concentrations of LH, P_4 , T and E_2

An autopsy at 73 hr after Pt treatment revealed that no ovulation took place until 65 hr from the treatment in the total 18 hens: 8 hens ovulated 67.5 ± 1.1 hr (mean $\pm$ s. e. m.) after the treatment and 10 hens did not ovulate up to the autopsy time. Two of the latter 10 hens showed a sign of the "bursting" atresia of the ovarian follicles. All the hierarchical yellow follicles of the ovary gained more weight as the time elapsed after Pt treatment in comparison to follicle weight of hens without Pt. The first, second and third largest preovulatory follicles (F1, F2 and F3) 73 hr after Pt treatment showed 102, 100 and 87% weight of the ovum ovulated a day before the Pt implant (average weight %, $N=7$). The weight of F1, F2 and F3 45 hr after Pt treatment was 96, 83 and 65% of the previously shed ovum while the weight of those follicles of hens without Pt 13.5 hr after C_2 ovulation showed 92, 79 and 54%, respectively.

Figure 2 illustrates the changes in plasma concentrations of LH, P_4 , T and E_2 up to 45 hr after Pt treatment in hens which did not ovulate. The plasma P_4 concentrations markedly increased soon after Pt treatment and remained high throughout the period ($P<0.01$). Plasma LH concentrations increased significantly 4 hr after Pt treatment ($P<0.01$) followed by a significant decrease ($P<0.01$) 45 hr after the treatment as compared to the pretreatment levels. Plasma T and E_2 concentrations also rose for the 2 hr and then decreased. Significantly lower levels, however, were detected at 45 hr ($P<0.01$).

Effect of LH Injection on Ovulation

The foregoing results may suggest either that the preovulatory LH surge is not released or that the ovary does not respond to spontaneous preovulatory LH surge. Accordingly, LH was injected to test the responsiveness of the ovarian follicle. As shown in Table 1, the in-

Fig. 2. Time course changes in plasma concentrations of LH (open circle), progesterone (closed circle), testosterone (open triangle) and estradiol (closed triangle) after implantation of Pt. Vertical lines indicate s.e.m. "a" indicate a significant difference from pretreatment levels at $P<0.01$. $n=7$ hens.

Table 1. Effect of Pt pretreatment on LH-, LHRH- and P- induced ovulation

Treatment	Number of Hens Injected	Number of Hens Ovulating	Interval* between Injection and Ovulation (h)
Experiment 2			
LH only	13	9	8.8 ± 0.9
Pt+LH	13	9	6.0 ± 0.4
Experiment 3			
LHRH only	10	6	8.4 ± 0.3
Pt+LHRH	10	8	4.7 ± 0.4
Experiment 4			
P only	8	6	8.6 ± 0.3
Pt+P	11	3	$4.0^+, 8.4^+, 10.5^+$

P and Pt indicate progesterone suspended in oil and progesterone in the Silastic tubings, respectively.

*, Intervals between injection and ovulation, are means $\pm$ s.e.m. in hens which ovulated before the autopsy time. The values with different superscripts (a, b) indicate a significant difference within each experiment (Student's t -test, $P<0.01$).

+, individual values.

duced-ovulation by LH injection in hens with Pt was the same rate as in hens without Pt. It is noted that the induction time was significantly shorter in hens with Pt implant than in hens without it ($P<0.01$).

Effect of LHRH Injection on Ovulation

LHRH was injected to test the responsiveness of the anterior pituitary. There was no

significant difference in the rates of induced ovulation between the hens with and without Pt (Table 1). The induction time was significantly shorter in those hens with Pt than in hens without Pt ($P < 0.01$).

Effect of P_4 Injection on Ovulation

Since the anterior pituitary gland responded to exogenous LHRH, P_4 was injected to study the responsiveness of the hypothalamus or other parts of CNS. In hens without Pt implantation, ovulation was induced in 6 of the 8 hens by P_4 injection and the induced-ovulation occurred at about the same rate as LH and LHRH injection. In contrast, in hens with Pt treatment ovulation was induced only in 3 hens and was not induced in other 8 hens P_4 injection.

Effect of LH Injection on Plasma Concentrations of LH, P_4 and T

Since there were considerable differences in LH, P_4 and T plasma levels at the time of LH injection between hens with and without Pt treatment. The hormone concentrations in plasma were expressed as the increment of the concentration after subtracting the prein-

Fig. 3. Increment of plasma concentrations of LH, progesterone and testosterone in response to LH injection in hens with either non-treated (closed circle) or treated (open circle) with Pt. In this subsequent figures, "a" indicate a significant difference from preinjection levels at $P < 0.01$. $n = 5$ hens.

jection value from the value at any given time. The hormone concentrations were measured only in hens which were induced ovulation by injection.

In hens subjected to LH injection without Pt treatment, there was no significant effect of LH injection on plasma LH concentrations (Fig. 3). Plasma P_4 concentrations significantly increased 2 hr after the injection and high levels were maintained. T levels rapidly increased and reached the plateau level 1 hr after the injection.

In hens with Pt implant, there was no difference of LH levels but plasma P_4 levels showed a faster increase when compared to hens without Pt treatment and the P_4 increments in Pt-treated hens were significantly larger than the hens without Pt ($P<0.01$). T concentrations in both groups increased significantly from preinjection levels ($P<0.01$).

Effect of LHRH Injection on Plasma Concentrations of LH, P_4 and T

Fig. 4 illustrates the time course changes in the increment of plasma LH, P_4 and T concentrations after LHRH injection. Plasma LH concentrations in hens without Pt markedly increased and attained a peak at 2 hr after the injection. In the Pt-treated hens, LH

Fig. 4. Increment of plasma concentrations of LH, progesterone and testosterone in response to LHRH injection in hens with either non-treated (closed circle) or treated (open circle) with Pt. $n=5$ hens.

increment was less prominent. The difference in the LH increment between two groups was significant ($P < 0.05$). Plasma P_4 concentrations significantly increased in hens without Pt after LHRH injection. Pt-treated hens showed higher levels of P_4 than non-treated hens ($P < 0.05$). Significantly higher levels of T were also maintained in non-treated hens whereas increased T concentrations immediately after the injection started decreasing 3 hr after the injection in hens with Pt treatment, which possibly corresponded to the latter half of the ovulation process (Table 1).

Effect of P_4 Injection on Plasma Concentrations of LH, P_4 and T

In hens with and without Pt, there was no significant increase in plasma concentrations of LH or T at any time after P_4 injection when compared to the respective preinjection levels (Fig. 5). On the other hand, the increment of plasma P_4 concentration was marked 1 hr after exogenous P_4 injection and continued increasing in hens without Pt. Greater P_4 increment may be attributable, at least in a part, to exogenous origin. As mentioned above (Table 1), ovulation was provoked by the injection only 3 out of 11 hens pretreated with Pt and the induction time was so variable that LH and steroid levels were

Fig. 5. Increment of plasma concentrations of LH, progesterone and testosterone in response to progesterone injection in hens with either non-treated (closed circle) or treated (open circle) with Pt. $n=5$ hens.

measured only in hens which did not ovulate. There was no statistical difference in the P_4 increments between the two groups.

Discussion

The present study demonstrated a prolonged inhibitory effect of continuous diffusion of P_4 from Silastic tubing on ovulation in the chicken. The delay of spontaneous ovulation for 41 hr reported here is much longer than that caused by a single P_4 injection²⁾. The delay may be attributable to lack of the preovulatory LH release. It is noted that basal LH levels were lowered 45 hr after the treatment to below pretreatment levels. Furthermore, the LH response to LHRH injection was significantly lower in the Pt-treated hens when compared to that in the non-treated hens, suggesting that exogenous P_4 partly act at the pituitary level as a negative feedback signal. It seems unlikely that this apparent suppression of LH release is merely reflected upon the empty of the LH store in the pituitary gland because the anterior pituitary seems capable of restoring LH production a few hours after stimulation according to WILSON and SHARP¹⁾. They demonstrated LH release by P_4 injection at any time during the ovulatory cycle after the preovulatory LH surge (an intrinsic stimulation). JOHNSON *et al.*¹⁶⁾ also showed significant rises in plasma LH concentration which were 1 hr apart in response to successive LHRH injections.

Among the steroid hormones in plasma, P_4 may serve as inhibitory signal in the regulation of the LH release because significantly higher levels were maintained throughout the experimental period. In contrast, T and E_2 concentrations in plasma did not increase significantly but decreased to those below the pretreatment levels. These steroid changes may be reflected by the LH levels in plasma or directly inhibited by exogenous P_4 . Although an inhibitory effect of large dose of E_2 on ovulations has been reported by FRAPS¹⁷⁾, their result is not pertinent to this study. It seemed that Pt stimulated LH release soon after the implantation but it suppressed LH release later. The switch mechanism, if any, from a positive to an inhibitory signal remains unknown. Positive feedback actions of P_4 is probably mediated by the hypothalamic P_4 receptors^{18,19)}. Prolonged treatment of Pt may alter characteristics of the hypothalamic P_4 receptor but nothing is known about possible roles of the P_4 receptor in relation to the negative feedback system.

Our data appear to support the idea that P_4 may exert its effect on the hypothalamus as well as other regions of the CNS which are implicated in the regulation of the preovulatory LH surge. First, after Pt treatment, the ovary responded to the exogenous LH challenge by a significant increase in plasma P_4 concentrations and the rate of induced ovulation was the same as that in hens without Pt treatment. In this case LH release might not be provoked by the rising levels of endogenous P_4 . Second, LHRH injection after Pt treatment also induced ovulation and the rate of the induced ovulation was larger than that in non-treated group. Although LH response was less prominent after LHRH stimulation in the Pt-treated hens when compared to that in hens without Pt treatment, it increased significantly 2 hr after the injection ($P < 0.01$), suggesting that the anterior pituitary is still responsive to its tropic hormone after Pt treatment. Third, an injection of P_4 in a dose sufficient to induce ovulation in intact hens failed to induce ovulation in the majority hens pretreated with Pt and no increase in plasma LH concentration was observed after P_4 injection.

Failure in inducing ovulation with P₄ injection in Pt-treated hens, on the other hand, may partly be attributable to modification of the ability of the hen after prolonged Pt treatments. Although the majority of Pt-treated hens did not ovulate in response to P₄ injection, the P₄ increments were not significantly different from those in non-treated hens which ovulated after P₄ injection. The high increments of P₄ levels in Pt-treated hens may be resulted from exogenous P₄ which half life might be prolonged by Pt treatment.

The shorter ovulation-induction time in hens pretreated with Pt in response to LH or LHRH injection was contrasted to hens without Pt treatment. The different responsiveness of the ovary to trophic hormones between hens with and those without Pt treatment may be explained not only by the more rapid release of endogenous P₄ but also by higher levels of P₄ in plasma after injections in hens with Pt than those without Pt.

The ovary after Pt treatment resembles that after PMSG treatment which disrupts the follicular hierarchy by enhancing growth of smaller follicles to the F1 size and leads to the cessation of ovulation²⁰⁾. Recent studies on the effect of PMSG on plasma levels of LH and steroid hormones demonstrate that plasma concentrations of P₄ and E₂ increased whereas the LH levels decreased after PMSG injections^{8,21-23)}. These studies suggests that P₄ and E₂ play a part in suppressing LH release after PMSG injections which delay spontaneous ovulations.

Summary

Progesterone in Silastic tubing (Pt) was implanted subcutaneously 1.5 hr after the second (C₂) ovulation of the sequence in the hen and effects of Pt treatment on subsequent ovulation and plasma concentrations of LH, progesterone (P₄), testosterone (T) and estradiol (E₂) were studied. Plasma concentrations of P₄ increased rapidly about 5-fold and remained high throughout 45 hr after Pt implantation. LH levels also significantly increased (1.4-fold) 4 hr after Pt treatment but declined thereafter to levels below the pretreatment levels. T and E₂ levels showed changes similar to LH levels but significant changes were detected only at 45 hr after the treatment. This treatment resulted in delayed ovulation for more than 41 hr. To examine the responsiveness of the hypothalamo-anterior pituitary-ovarian axis in those hens either ovine LH (100 µg), synthetic [Arg⁸]-LHRH (25 µg) or P₄ (1.0 mg) was injected at 45 hr after Pt implantation. Both LH and LHRH injections induced ovulations at the rate of 70 and 80%, respectively. Conversely, P₄ injection (after Pt treatment) failed in inducing ovulation in the majority of hens examined (ovulation, 27%). The same of ovine LH, LHRH and P₄ induced ovulations at the rates of 70, 80 and 75%, respectively, when they were injected 13.5 hr after C₂ ovulation in hens without Pt treatment. These results suggest that exogenous Pt may exert its inhibitory effect on the hypothalamus or other regions of the CNS implicated with regulation of the preovulatory LH release. The ovulation-induction time in hens pretreated with Pt was shorter in response to LH (6.0 hr) or LHRH (4.7 hr) injection when compared to hens without Pt treatment (8.8 hr). This was reflected upon higher concentrations of plasma P₄ in Pt-treated hens.

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プロゲステロン持続性投与の排卵抑制効果

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ニワトリの LH 分泌に対する プロジェステロン (P_4) の正のフィードバック作用については多数の報告があるが負のフィードバック作用についての研究はほとんどなされていない。従って, 本研究は, 人為的に血中 P_4 濃度を高濃度に維持し, 次卵排卵の抑制効果と血中 LH, P_4 , テストステロン (T) およびエストラジオール (E_2) 濃度におよぼす影響と, さらに, 視床下部一下垂体前葉一卵巣軸のどのレベルで抑制作用が起こるのかを検討するために行なった。

10-14 ケ月齢のニワトリ (1.55-2.21 kg) を個別ケージに入れて, 飼料と水は自由摂取, 照明は 14 時間/日のもとで飼育した。放卵記録装置により産卵時刻を毎日記録し, クラッチサイズが 5-8 卵からなり, クラッチ間休産日が一日で比較的規則正しい産卵パターンを示すニワトリを実験に使用した。

クラッチ第 2 卵 (C_2) の排卵後 1.5 時間にサイラスチックチューブ (長さ 5cm, 内径 2.64 mm) に充填したプロゲステロン粉末 (Pt, 2 本/1 羽) をニワトリの腹部皮下に植え込んだ。排卵時刻は全ての実験において予想される時刻の 2-4 時間後に剖検を行ない排卵した卵の卵管内位置から推定した。

実験 1. Pt 処理後 45 時間と 73 時間にニワトリを殺し排卵時刻, 血中ホルモン動態, 卵胞重量を測定した。実験 2. 処理群には Pt 処理後 45 時間にヒツジ LH (100 μ g) を, 未処理群には比較として Pt 処理しないニワトリの C_2 排卵後 13.5 時間 (C_3 排卵前 12 時間) に LH (100 μ g) を投与した。実験 3 と 4 では実験 2 と同様な時間に合成 [Arg⁸]-LHRH (25 μ g) あるいは P_4 (0.1 mg) をそれぞれ投与した。

採血は上腕静脈にカテーテルを取り付け, 実験 1 では Pt 処理後 0, 2, 4, 6 と 45 時間に, 実験 2, 3, 4 では Pt 処理, 未処理群とも LH, LHRH あるいは P_4 投与の 0,

1, 2, 3, 4 時間に連続的に行ない, 血漿を -20°C に保存した。LH, P_4 , T および E_2 の血中濃度はラジオイムノアッセイにより行なった。

Pt 処理により血中 P_4 濃度はすみやかに増加し 45 時間にわたって高い濃度が維持された。LH 濃度も植え込み後 4 時間までは増加したが, その後は植え込み前の値より低下した。T と E_2 濃度も LH 濃度と同様な変化であったが有意差が見られたのは植え込み後 45 時間のみで最低濃度となった時であった。また Pt 処理により次卵排卵は 41 時間以上遅延した。すなわち, この間には, もし Pt 処理しなければ次卵 (C_3) 排卵はもとより C_4 排卵も起こったはずである。

Pt 処理後の視床下部一下垂体前葉一卵巣軸の反応性を検討するために Pt 処理後 45 時間に LH, LHRH あるいは P_4 を投与した結果, 前二者に対して排卵は 70, 80% の率で誘起されたが P_4 に対しては 27% 誘起されたにすぎなかった。なお, Pt 未処理群ではそれぞれ 70, 60, および 78% の排卵誘起率であった。また, 排卵は Pt 処理群の方が未処理群より LH および LHRH 投与後早く誘起された。

Pt 処理後の LH あるいは LHRH 投与に対し血中 P_4 濃度は未処理群よりも早く増加し, 測定期間 (4 時間) 全体にわたって高い値に維持された。また, Pt 処理群の卵胞重量は未処理群より大きかった。

以上の結果, Pt 処理によって排卵が誘起されるのは P_4 が視床下部を含む脳神経系に作用して LHRH 放出を抑制しているからであろうと考えられた。また Pt 処理後による排卵反応性 (排卵誘起率, 排卵誘起時間) の増加は, 処理後の卵巣の反応性 (P_4 分泌) の増加に関連していると考えられた。

(家禽会誌, **23** 巻, 211~221, 1986)