



STANDARD ARTICLE

Relationship between endogenous plasma adrenocorticotrophic hormone concentration and reproductive performance in Thoroughbred broodmares

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Abstract

Background: Pituitary pars intermedia dysfunction (PPID) is an endocrine disorder the clinical signs of which include infertility, but few reports have examined the relationship between PPID and reproductive performance in broodmares.

Hypothesis/Objective: Broodmares with infertility of unknown etiology were screened for PPID using baseline endogenous plasma ACTH concentrations, and its relationship with subsequent reproductive performance was analyzed.

Animals: Sixty-seven Thoroughbred broodmares.

Methods: Adrenocorticotrophic hormone concentrations were measured in autumn. The broodmares were divided into 3 groups based on endogenous plasma ACTH concentration (low, <50 pg/mL; intermediate 50-100 pg/mL; and high >100 pg/mL) as recommended by the Equine Endocrinology Group. We compared these results with those of other blood tests, clinical examinations, subsequent conception, and frequency of normal delivery.

Results: The numbers of horses per group (percentage) were 22 (32.8%), 20 (29.9%), and 25 (37.3%). The mean \pm SD ages (years) were 11.1 ± 3.4 , 12.1 ± 4.5 , and 13.0 ± 4.3 , with no significant difference among groups. Mean \pm SD plasma cortisol concentrations ($\mu\text{g/dL}$) were 4.0 ± 1.6 , 7.1 ± 1.4 , and 7.6 ± 1.9 , with the intermediate and high ACTH groups having significantly higher results than the low ACTH group ($P < .001$). Conception rates were 77.3%, 89.5%, and 56.5% and normal delivery rates were 77.2%, 84.2%, and 43.5% with the percentage of each significantly lower in the high ACTH group ($P = .02$, $P = .008$, respectively).

Conclusions and Clinical Importance: The study group of infertile broodmares may include horses with PPID. The reproductive performance of the high ACTH group was significantly lower than the other groups. Early diagnosis and treatment of infertile broodmares by screening plasma ACTH concentrations could help improve reproductive performance.

Abbreviations: BCS, body condition score; ID, insulin dysregulation; MSH, alpha-melanocyte-stimulating hormone; PPID, pituitary pars intermedia dysfunction.

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KEYWORDS

ACTH, broodmare, ECD, fertility, horse, PPID, pregnancy

1 | INTRODUCTION

Pituitary pars intermedia dysfunction (PPID) is disease of horses associated with hyperplasia of pituitary pars intermedia cells and caused by decreased dopamine secretion from the neurons that regulate them. This condition is similar to Cushing's syndrome, which affects other species, but the mechanism that causes these abnormalities in horses is not clear.¹⁻⁶ Signs may be related to functional deterioration stemming from physical compression of the pars distalis, pars nervosa, and hypothalamus which is associated with hyperplasia of the pars intermedia and abnormal secretion of related hormones, including α -melanocyte stimulating hormone (α -MSH), adrenocorticotrophic hormone (ACTH), dopamine, and cortisol.⁷⁻⁹ Characteristic clinical signs of advanced PPID are hypertrichosis and laminitis.⁹⁻¹³ In addition, other signs are reported such as weight loss, pendulous abdomen, polydipsia, polyuria, reproductive disorders, and respiratory and urinary tract infections associated with altered immunity.^{1,3,9}

In the past, the 19-hour overnight dexamethasone suppression test has been recommended for PPID diagnosis.^{1,14} In recent years, however, measurement of endogenous plasma ACTH concentrations has been recommended.^{8,15} Plasma ACTH concentrations display seasonal fluctuation, with higher concentrations observed from summer to autumn.^{15,17} Therefore, it is recommended to evaluate concentrations according to seasonally adjusted reference ranges so as to optimize diagnosis of PPID.¹⁵

As broodmares age, they have decreased conception and delivery rates,^{18,19} but some older Thoroughbred broodmares have highly regarded pedigrees and economic value, and maintaining their fertility is desirable. Some factors contributing to a lower conception rate in older broodmares are age-related physical changes of the uterus and vagina, as well as trauma related to delivery.^{18,19} The hypothalamic-pituitary-adrenal axis also can be compromised by infectious diseases such as endometritis and by endocrine dynamics related to reproduction.^{3,20} Pituitary pars intermedia dysfunction is an age-related endocrine abnormality reported to occur in 15% to 30% of horses over the age of 15 years.^{1,9} Early diagnosis and treatment of PPID may prevent or minimize related complications.^{13,21,22} Little research has been performed describing the relationship between PPID and reproductive performance, conception rates, and delivery rates.^{20,23,24} In Japan, no studies have focused on PPID in Thoroughbred broodmares, and no literature on diagnosis and treatment is available.^{25,26} Without such guidance, negative economic impact is likely for owners whose aging broodmares have decreased fertility and a shortened reproductive period.

Our purpose was to investigate the influence of PPID and high endogenous plasma ACTH concentrations on reproductive performance, including conception rate and normal delivery rate in broodmares that had previously failed to conceive.

2 | MATERIALS AND METHODS**2.1 | Animals**

Sixty-seven Thoroughbred broodmares (4-22 years old; average age, 12 years; median age, 11 years) were evaluated. These mares were managed and bred at multiple breeding farms in the Hidaka district of Hokkaido, Japan, had not conceived in the 2017 breeding season, and were presented at veterinary clinics in the autumn to be examined for failure to conceive. None of the horses had been treated with pergolide or other medications.

2.2 | Sample collection, processing, and storage

From October 30 to November 13, 2017, morning blood samples were collected from the jugular veins of infertile mares. For measurement of plasma ACTH concentrations, 5-mL blood samples were collected using serum separator tubes (Venoject II with EDTA-2Na, Terumo, Tokyo, Japan). For measurement of cortisol and insulin concentrations, 10-mL blood samples were collected using tubes containing a serum separator (Venoject II with Serum Separator medium). For measurement of blood glucose concentrations, 5-mL blood samples were collected using sodium fluoride heparin Na and EDTA-2Na-coated plasma collection tubes (Venoject II with sodium fluoride, Heparin Na, EDTA-2Na). Samples were immediately placed in a cooler box containing an ice pack and transported to the General Clinical Laboratory Center (Sapporo Clinical Laboratory Center Co, Ltd) on the same day.²⁷

2.3 | Assays

Plasma ACTH concentration was measured using a chemiluminescent enzyme immunoassay (Immulite2000, Siemens Healthcare Diagnostics, Inc, Tokyo, Japan) with a commercially available enzyme immunoassay (EIA) kit widely used for animal species (Siemens Immuryze ACTH II, Siemens Healthcare Diagnostics Inc) for plasma ACTH concentrations in the range of 10 to 1250 pg/mL.²⁸ The average coefficient of variation measured for 10 samples was 0.04%.

Plasma cortisol concentration was measured by chemiluminescent enzyme immunoassay (Unicel DxI800, Beckman Coulter Inc, Tokyo, Japan) using a commercially available EIA kit widely used in animal species (Access Cortisol, Beckman Coulter Inc) for plasma cortisol concentrations in the range of 0.4 to 60 μ g/dL.^{20,29} The average coefficient of variation measured for 10 samples was 0.04%.

Insulin was measured using a chemiluminescent enzyme immunoassay (Lumipulse L2400, Fujirebio, Tokyo, Japan) and commercially

available EIA kit widely used in animal species (Lumipulse Presto Insulin) for plasma insulin concentrations in the range of 0.1 to 400 $\mu\text{IU/mL}$.^{13,30} The average coefficient of variation measured for 10 samples was 0.04%.

Blood glucose concentration was measured using the glucose oxidase colorimetric method (JCA-BM9130, Nihon-Denshi, Tokyo, Japan) with a commercially available glucose oxidase assay kit widely used in animal species (DETAMINAR L Glu HK, Nipro Corporation, Osaka, Japan) for blood glucose concentrations in the range of 5 to 1000 mg/dL.¹ The average coefficient of variation measured for 10 samples was 0.01%.

2.4 | Classification by endogenous plasma ACTH concentration

Based on the criteria recommended by the Equine Endocrinology Group for PPID diagnosis, the broodmares were divided into 3 groups based on results of endogenous plasma ACTH concentrations: Low ACTH (<50 pg/mL), intermediate ACTH (50-100 pg/mL), and high ACTH (>100 pg/mL).³⁰

2.5 | Analysis of breeding data

We collected data from the 2018 spring breeding season, evaluating the mating and conception patterns of the broodmares; delivery status in the spring of 2019 was extracted from the database of the pedigree service of the Japan Association for International Racing and Stud Book.

The number of mating broodmares is the cumulative total number that mated during 2018. The conception rate was the number of successful conceptions through the end of 2018, except those that ended in early embryonic death. The normal delivery rate was defined as the ratio of broodmares that delivered normally divided by the total number of broodmares excluding early embryonic deaths, immediate postnatal deaths, stillbirths, and mortality of broodmares mares during pregnancy. We also compared the relationship among ACTH, cortisol, insulin, blood glucose, and clinical examination findings with the number of mating, conception rates, and normal delivery rates.

2.6 | Clinical examination

Clinical examinations were performed in the autumn of 2017 (October 30 to November 13) after blood sampling. Hypertrichosis characteristics of PPID, hoof abnormalities, and body condition score (BCS) were recorded.^{10,12,31} Abnormalities of the uterus, vagina, and ovaries were evaluated by palpation and ultrasonographic examination during rectal palpation, and injuries to the vagina and external cervical ostium were evaluated by transvaginal palpation and endoscopic examination.

Cytological examination of uterine lavage fluid was performed as follows: 50 mL of sterile saline was infused into the uterus using an

endoscope; the lavage fluid was collected, and bacterial and cytological tests were performed.³² In sample analysis, the presence of neutrophils indicated the presence of inflammation. For histological examination of the endometrium, an endometrial biopsy specimen was collected under endoscopic guidance using biopsy forceps. Inflammatory cell infiltration and degenerative changes in the uterine glands were diagnosed by histopathological analysis. The grading system used was based on that developed previously³³: mares classified as grade I had a normal endometrium or mild, focal inflammation or fibrosis; grade IIa mares had mild to moderate inflammation of the endometrium with or without multifocal areas of periglandular fibrosis with 1 to 3 layers of fibroblasts surrounding glands or an average of <2 fibrotic glandular nests per 5 mm linear field; grade IIb mares had multifocal areas of moderate inflammation with or without multifocal fibrosis with ≥ 4 layers of fibroblasts surrounding the glands or an average of 2 to 4 fibrotic glandular nests per 5 mm linear field; and grade III mares had severe inflammation with or without diffuse fibrosis with an average of ≥ 5 fibrotic glandular nests per 5 mm linear field.

2.7 | Statistical analysis

Statistical analysis was performed using commercial statistical software (JMP 14.2, SAS Institute, Inc, Cary, North Carolina), and *P* values <.05 were considered significant. Comparisons among groups for age, ACTH, cortisol, insulin, blood glucose concentration, and BCS were performed using the Turkey-Kramer honestly significant difference (HSD) test after utilizing 1-way analysis of variance to analyze the mean value. Furthermore, for ACTH and cortisol, a correlation coefficient was calculated after confirming the normal distribution of both data sets.

Pearson's Chi-squared tests were used to compare groups for the incidence of abnormal external cervical ostia, high grades of endometrial histology, incidence of inflammatory cells (including neutrophils) in uterine lavage fluid, mating frequency, conception rates, and normal delivery rates.

3 | RESULTS

Table 1 shows the number and proportion of broodmares in each of the 3 study groups (low, intermediate, and high ACTH), average age (years), ACTH concentrations (pg/mL), cortisol concentrations ($\mu\text{g/dL}$), insulin concentrations ($\mu\text{IU/mL}$), blood glucose concentrations (mg/dL), BCS, and various reproductive performance indices (number of matings, conception rates, and normal delivery rates). No significant difference existed in the average age among the groups (between high and low ACTH; *P* = .28).

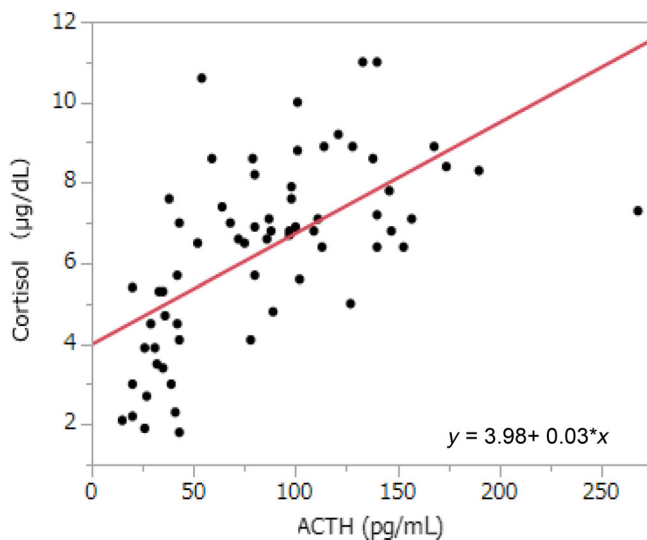
The ACTH concentrations were significantly different among groups (*P* < .001). The cortisol concentrations were significantly higher in the intermediate and high ACTH groups than in the low ACTH group (*P* < .001). The correlation coefficient between ACTH and cortisol concentrations was 0.61 (*P* < .001; Figure 1). Insulin and blood glucose

TABLE 1 Phenotypic markers, endocrine values, and management factors in a population of subfertile Thoroughbred broodmares (n = 67)

Variable	Low	Intermediate	High
Mare (n)	22	20	25
Proportion of all mares (%)	32.8	29.9	37.3
Age (years)	11.09 ± 3.44	12.10 ± 4.53	13.00 ± 4.34
ACTH (pg/mL)	32.55 ± 8.49 ^{ab}	79.05 ± 13.84 ^{ac}	139.92 ± 36.20 ^{bc}
Total cortisol (µg/dL)	3.99 ± 1.57 ^{ab}	7.05 ± 1.36 ^a	7.64 ± 1.88 ^b
Insulin (µIU/mL)	10.14 ± 7.40	10.94 ± 6.47	12.50 ± 17.05
Glucose (mg/dL)	98.86 ± 23.51	94.20 ± 14.11	91.56 ± 13.04
BCS	5.60 ± 0.53	5.53 ± 0.60	5.50 ± 0.58
Average number of matings	1.27 ± 0.45	1.42 ± 0.82	1.48 ± 0.71
Number of conceptions (n)	17	17	13
Conception rate (%)	77.3	89.5 ^c	56.5 ^c
Number of normal deliveries (n)	17	16	10
Normal delivery rate (%)	77.3 ^b	84.2 ^c	43.5 ^{bc}

Notes: Values are reported as mean ± SD. Significant differences ($P < .05$) were shown between the same letter (a, b, c).

Abbreviation: BCS, body condition score.

**FIGURE 1** Correlation between plasma ACTH and serum cortisol concentrations. The correlation coefficient is 0.61 ($P < .001$)

concentrations were not significantly different among the groups. No significant difference was found in mean BCS among the 3 groups.

The average number of matings was higher in the high ACTH group, but this difference was not statistically significant. Table 2 shows the conception and normal delivery rates for each group. In the high ACTH group, the conception rate was significantly lower than in the intermediate ACTH group ($P = .02$). The normal delivery rate was significantly lower in the high ACTH group than in the low and intermediate ACTH groups ($P = .008$).

Physical examinations found all horses to be healthy, and no horse had obvious abnormalities in appearance, including hair coat and hooves. No horse had obvious physical abnormalities of the uterus, vagina, or ovaries. Table 3 shows the number of injuries to

the vagina and external cervical ostia, cytological examination findings of the uterine lavage fluid, and histological examination findings of the endometrium. The number of injured vaginal and external uterine ostia was significantly higher in the high ACTH group than in the low ACTH group ($P = .03$). No significant difference was found in the frequency of neutrophils in the uterine lavage cytology among the 3 groups. Endometrial histology showed no significant differences in grades among the groups.

4 | DISCUSSION

The incidence of high ACTH concentrations is high in older horses, and it increases with age, with few reports in horses <10 years old.^{1,3,7} The horses in our 3 study groups were not significantly different in average age. Based on our results, almost two-thirds of this population of subfertile broodmares that have high ($n = 25, 37.3\%$) or intermediate ($n = 20, 29.9\%$) ACTH concentrations might have PPID based on high endogenous plasma ACTH concentrations, which represents a much higher percentage than reported in the general equine population.^{1,9} The numbers of horses that had suspected PPID were higher than previously reported because we only tested mares that had reproductive disorders (infertility), which is considered a clinical sign of PPID. Some individuals incidentally may have had high ACTH concentrations in the high and intermediate groups. Younger horses also might be affected by PPID.

Plasma ACTH concentrations have been reported to be increased in PPID-affected horses, but no significant difference was observed in plasma cortisol concentrations,^{29,30} and because ACTH is produced in the pars intermedia from proopiomelanocortin, can be active or inactive. When the total amount of active and inactive ACTH is increased, the plasma ACTH concentration is high. However, because much of circulating ACTH can be inactive, it might not influence

TABLE 2 Descriptive data of number and rate of conception, no conception, normal deliveries, death after birth, and spontaneous mare death in all mares (n = 65)

Variable	Number of conception (n)	Conception rate (%)	Number of no conception (n)	No conception rate (%)	Number of normal deliveries (n)	Normal delivery rate (%)	Number of direct death or stillbirth (n)	Rate of direct death after birth or stillbirth (%)	Number of spontaneous mare death (n)	Rate of spontaneous mare death (%)
Low	17	77.27	5	22.73	17	77.27 ^b	0	0.00	0	0.00
Intermediate	17	89.47 ^a	2	10.53	16	84.21 ^c	1	5.26	0	0.00
High	13	56.52 ^a	10	43.48	10	43.48 ^{bc}	2	8.70	1	4.35

Notes: Significant differences ($P < .05$) in conception and normal delivery rate were shown between the same letter (a, b, c). Each unmated mare in intermediate and high ACTH groups were excluded (n = 2).

TABLE 3 Descriptive clinical examination data in all mares (n = 67)

Variable	Grade	Low	Intermediate	High
Mare (n)		22	20	25
Number of injuries of vagina and cervix (n)		0*	1	5*
Number of abnormalities of cytological examination of uterine lavage fluid (n)		7	4	7
Number of abnormalities of endometrial histology (n)	2A	8	13	1
	2B	10	9	1
	3	10	13	1

Notes: Values are reported indicate absolute number of occurrence. Grade of endometrial histology was based on a previous report.³³

*Significant difference in conception rate was shown between low and high ACTH groups ($P < .05$).

cortisol secretion in proportion to its plasma concentration.⁸ In our study, however, although the concentrations of cortisol were not increased to the magnitude that can be observed in association with high intensity exercise, the high and intermediate groups had significantly higher concentrations than did the low ACTH group ($P < .001$).³⁴ We also found a correlation between ACTH and cortisol (correlation coefficient = 0.61; $P < .001$).

Most previous studies have concluded that plasma cortisol concentration does not increase in PPID, and thus the reason for the increase in cortisol concentrations in our study is unclear. However, we did not target PPID horses, but limited evaluation to infertile broodmares. Therefore, it is possible that other hormonal abnormalities affected by stimulation of the pars distalis, including cortisol, could have affected reproductive function. Because the pars distalis is involved in the release of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and prolactin related to reproduction, it also may affect the dynamics of these hormones.

Furthermore, a report showed that a short-term increase in plasma cortisol concentrations associated with stress, such as a single examination or transportation, does not affect reproductive performance,²⁰ but no studies have evaluated long-term effects.

Therefore, when plasma ACTH and cortisol concentrations continue to increase for a long period of time, secretion of reproductive hormones, including FSH, LH and prolactin that are related to compromised tissue and follicle development, may be affected.³⁰

No significant differences were found in insulin and blood glucose concentrations among the groups ($P > .05$). Many horses with PPID also have insulin dysregulation (ID), and high insulin concentrations are seen simultaneously.^{3,13} A relationship between ID and reproductive disorders has been reported.²⁴ In the broodmares of our study, however, no significant difference was found in BCS among the groups, and no broodmares had characteristics such as obesity and laminitis that are typical of ID associated with equine metabolic syndrome. In our study group, 3 horses had insulin concentrations ≥ 20 $\mu\text{U}/\text{ml}$ in the positive group (20.4, 22.4, and 90.6 $\mu\text{U}/\text{mL}$), 2 horses in the suspect group (22.6 and 28.1 $\mu\text{U}/\text{mL}$) did, and 2 horses in the negative group (25.5 and 29.3 $\mu\text{U}/\text{mL}$) did. Therefore, although the potential complication of ID in those small numbers cannot be denied, the insulin concentrations in the other horses were within the normal range, and none of the groups showed significant differences in insulin and blood glucose concentrations. From our study results, it is probable that high ACTH and PPID and not ID are more likely to contribute to reproductive failure such as sterility.

From our clinical examination results, only the injuries to the external cervical ostia were significantly higher in the high ACTH group than in the low ACTH group ($P = .03$), but no significant differences were found in uterine lavage cytology findings and endometrial histopathology. The reason for the high rate of external cervical ostium injury in the high ACTH group is not clear, but the low conception rate inevitably increases the number of matings and proportion of

abnormalities during delivery, such as dystocia and postnatal death. It is suspected that the probability of injury because of physical factors was high.

Although the injury rate of the external cervical ostium was significantly higher in the high ACTH group, no significant differences were found in the histopathological results of the endometrium and in uterine lavage cytology. It is believed therefore that these have less influence on reproductive performance.

The horses in the high ACTH group did not have significant differences in the number of matings, but horses in the intermediate and high ACTH groups had lower rates of conception ($P = .02$), and the normal delivery rate was significantly lower in the high ACTH group ($P = .008$). The low conception and delivery rates in the high ACTH group may be a consequence of increases in cortisol concentration because of high ACTH concentration that could make it easier for uterine and other infections to occur. Although the mechanism is unclear, abnormal secretion of reproductive hormones from the hypothalamus and pituitary glands, such as FSH, LH, and androgens, may have affected ovulation, fertilization, and maintenance of pregnancy.^{23,24,28}

5 | CONCLUSION

We showed that many infertile mares of all ages had high plasma ACTH concentrations. Furthermore, high ACTH concentrations may indirectly affect reproductive performance, such as conception and delivery, because of abnormal secretion of various reproductive hormones, including ACTH and cortisol. This result suggests that early diagnosis of infertile mares using endogenous plasma ACTH concentration may allow improved reproductive performance.^{8,21,35}

Because the number of animals in our studies was relatively small (67) and of a single breed, and the external conditions such as feeding management were not the same, other factors (including other reproductive hormones) should be investigated in addition to the effects of high ACTH or PPID on reproductive performance.

It will be necessary to carry out additional studies utilizing large farms with larger numbers of animals, consistent feeding practices, and well-managed breeding programs to further evaluate our findings.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

This study was done according to the Ethical Principles in Animal Experimentation and approved by the Ethics Committee of the Institutional Animal Welfare and Experiment Management Committee of the Hidaka Training and Research Center (#2017-1).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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