Adrenocortical Response in Cows after Intramuscular Injection of Long-Acting Adrenocorticotropic Hormone (Tetracosactide Acetate Zinc Suspension)

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Introduction

Stress is one of the important factors adversely affecting the health and productivity of dairy cows. The degree of acute stress can be estimated by the elevation of plasma cortisol concentrations (Tarrant et al. 1992). It is also needed to know whether the stress response causes any alteration of adrenocortical function.

The adrenocortical function has been tested by a rapid ACTH challenge (Gwazdauskas et al. 1980; Nakao and Grunert 1990; Yoshida and Nakao 2005), in which ACTH was injected intramuscularly and plasma cortisol concentrations were measured before and 0.5–2 h after the challenge with ACTH.

Although a rapid ACTH test based on plasma cortisol concentrations is a reliable method to evaluate adrenocortical function, it is not practical to implement when a large number of cows on commercial dairies are to be used. It would be very practical and useful if an ACTH challenge could be conducted based on cortisol concentrations in milk collected at a routine milking time, i.e., morning and afternoon. The collection of milk samples at routine milking time may not add any stress in the animals and may not require extra time and labour for the sampling. The response of plasma cortisol after ACTH should be continued for approximately 8–12 h, if ACTH is injected after morning milking and the second milk samples are collected before afternoon milking.

Use of a short-acting ACTH, therefore, is not suitable for this type of ACTH test. It may be necessary to use a long-acting ACTH-Z to evaluate adrenocortical response at afternoon milking.

The aims of this study were, therefore, to show adrenocortical response to ACTH-Z and its effect on adrenocortical function in beef cows and to know the response of cortisol in milk in comparison with plasma cortisol 8 h after an ACTH-Z challenge.

Materials and Methods

Experiment 1

This experiment was carried out at Yamaguchi University Experimental Farm in Sep 2009. Four non-suckling beef cows (two Japanese Black beef cows and two Japanese Black and Holstein Friesian crossbred cows) were used for this experiment. Age of cows ranged from 10 to 14 years, and they were one and half years post-partum or longer. The mean body weight was 513 ± 44 kg (range: 447–637 kg). All handling of the animals was performed with a minimal disruption of their daily routine. Cows were able to lie down, eat and drink ad libitum. The experimental protocol and animal care during the experiment accorded with the International Guide Principles for Biomedical Research Involving Animals (Council for International Organizations of Medical Sciences).

Oestrus was synchronized in all cows utilizing CIDR-Heatsynch protocol (Yusuf et al. 2008). Indwelling jugular vein catheters were inserted 6 h prior to each of the ACTH challenge. On day 7 to 10 of oestrous cycle (day 0 is day of oestrus), the cows were challenged with 0.5 mg tetracosactide acetate zinc suspension (ACTH-Z; Controsyn²Z, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan) per head. ACTH-Z is tetracosactide acetate zinc suspension. A dose of 0.5 mg has been known to have an action to stimulate the adrenal cortex equivalent to 20 IU natural ACTH-Z.

BLOOD samples were collected at −1, −0.5, 0 (immediately before the ACTH-Z injection), 0.5, 1 h and every 2 h up to 36 h and at last 48 h after ACTH-Z injection.

A rapid ACTH challenge was conducted 3 days before ACTH-Z treatment and also 2 h after the
completion of ACTH-Z challenge for 48 h. Cows were injected intramuscularly with 0.25 mg tetracosactide acetate in physiological saline (Controsyn®; Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan). It has been reported that 0.25 mg of ACTH (tetracosactide acetate) is equivalent to 25 IU natural ACTH. Blood samples were taken at –1, –0.5, 0 (immediately before the ACTH injection), 0.5, 1, 2, 4 and 6 h after ACTH stimulation.

Blood samples were immediately stored at 4°C and centrifuged (1500 x g for 15 min) within 30 min after collection. Plasma was kept at –20°C until analysis for cortisol.

Experiment 2
This experiment was carried out at Niigata University Experiment Farm during a period from Apr 2009 to Feb 2010. Eight lactating dairy cows in their first to sixth lactation were used in this experiment. Their body weight varied from 572 to 848 kg (mean 704.9 ± 95.8 kg).

The cows were randomly assigned into two groups. A group of six cows received 0.5 mg ACTH-Z 1–2 weeks post-partum, while the other group of two cows received 0.5 ml saline solution. All the cows were treated again in the same manner 4–5 weeks after calving.

In both groups of cows at both post-partum periods 1–2 weeks and 4–5 weeks post-partum, blood and milk samples were collected three times within 5 min before the ACTH-Z injection, at the afternoon milking on the same day and before the morning milking on the following day. Blood was taken by tail venepuncture into heparinized tubes, and plasma was collected after centrifugation at 1500 x g for 15 min. Milk sample was preserved by potassium dichromate and then centrifuged at 1500 x g for 4°C for 15 min. Plasma and skim milk were frozen until assayed.

Cortisol assay
Plasma cortisol assay
Plasma cortisol concentrations were determined by an enzyme immunoassay established and validated by Yoshida and Nakao (2005). The intra-assay and inter-assay coefficients of variation in high and low cortisol pooled plasma samples were 7.6 % (n = 5) and 10.5 % (n = 5) and 7.1 % (n = 5) and 7.7 % (n = 5), respectively.

Skim milk cortisol assay
The cortisol concentrations in skim milk were determined after extraction with dichloromethane as reported by Waki et al. (1987). Cortisol was extracted two times from skim milk (100 µl) with 2.0 ml dichloromethane (Kanto Chemical Co. Inc., Tokyo, Japan) in a glass tube. The mixture was shaken at 250 x g for 15 min in a shaker (Vial Mixer Vix-100, Taitec, Japan), and the supernatant solution was transferred to another glass tube. The extracted solution was evaporated by heating in a hot water bath (50°C) for 2 h. After complete drying, 100 µl assay buffer, 100 ml borate buffer (boric acid 1.55 g + distilled H₂O 500 ml) added with 0.2 g bovine serum albumin (BSA; Sigma-Aldrich, Japan) and 0.01 g thimerosal (sodium ethylmercurithiosalicylate; Nacalai Tesque, Inc., Kyoto, Japan) were put into the tube and mixed by shaker for 10 min.

Wells of the microtitre plate (Sumilon, Sumimoto Bakelite Co. Ltd., Japan) were coated with 100 µl of 4 µg/ml anti-rabbit IgG antibody (Cosmo-Bio, Japan) in carbonate buffer (0.05 M Na₂CO₃ and 0.05 M NaHCO₃ in distilled water mixed at 2 : 5 ratio and pH adjusted at 9.7), incubated at room temperature for 1 day, washed three times with 0.9% NaCl solution, blocked with 200 µl of blocking solution (100 ml of 0.05 M phosphate buffer + 0.1 g BSA + 3 g sucrose) and incubated at room temperature for 1 h. The solution in the wells was removed. After drying, plates were kept in a refrigerator.

Fifty microlitres of 0, 0.1, 0.3, 1, 3, 10 and 30 ng/ml cortisol (Sigma Reference Standard, Sigma-Aldrich Chemie, Germany) standard solutions in assay buffer, or sample, were added into the wells. To these, 50 µl of diluted cortisol-3-CMO-BSA-HRP (4000 times final dilution, Kambegawa Institute, Japan) was added, and then 50 µl of diluted anti-cortisol-3-CMO-BSA IgG, 25 000 times at final dilution (Kambegawa Institute, Japan) was added. The plate was sealed, shaken well and incubated at room temperature for 3 h. After incubation, the wells were washed three times with phosphate buffered saline (PBS). Then, 150 µl of 4 mg/ml OPD (o-phenylenediamine; Nacalai Tesque, Inc.) in 0.2 M citric acid (pH 4.5) and 0.02% H₂O₂, was added. This was followed by incubation at room temperature in dark for 1 h. The reaction was stopped by the addition of 50 µl of 6N H₂SO₄. The plate was shaken well, and the optical density was measured at 492 nm in a microplate reader (Sunrise-Basic Tecan, Austria).

The intra-assay and inter-assay coefficients of variation in high and low cortisol were 7.8 % (n = 6) and 16.3 % (n = 6), and 2.7 % (n = 4) and 14.2 % (n = 4), respectively.

Statistical analysis
All analyses were performed using srs 16.0 for Windows (SPSS InC., Chicago, IL, USA). The data obtained from Experiment 1 and 2 were analysed for normal distribution. The results in Experiment 1 were not normally distributed. Therefore, results from Experiment 1 were presented as median and interquartile range. The Kruskal–Wallis test was used to analyse the differences in cortisol concentrations by different time after treatment. When the difference by time after treatment was significant (p ≤ 0.05), the differences between the basal and responding values of plasma cortisol concentrations were analysed using Wilcoxon’s test. In Experiment 2, results were presented as the mean ± standard error (±SE), because the data were normally distributed. The differences in the mean values of plasma and skim milk cortisol concentrations between ACTH-Z and control groups at different times were compared by repeated measures. After confirming the significant effects of treatments, times and their interaction, multiple comparison tests
were conducted to find the difference among treatments and times using two-way analysis of variance. The relationship between cortisol concentrations in plasma and skim milk in Experiment 2 was analysed by correlation analysis ANOVA.

**Results**

**Response of plasma cortisol in beef cows after ACTH-Z challenge**

Median plasma cortisol concentrations increased drastically at 30 min and reached a peak of 34.9 ng/ml at 4 h after ACTH-Z injection. The cortisol values then decreased gradually but were still high until 10 h after ACTH-Z injection (Fig. 1).

All the cows had normal adrenocortical function before the ACTH-Z challenge, which did not affect the adrenocortical function as shown by a high response to ACTH (Fig. 1).

**Adrenocortical function based on ACTH-Z challenge in post-partum dairy cows**

All the cows showed a significant increase in cortisol concentrations in plasma as well as in skim milk 8 h after ACTH-Z injection 1–2 weeks and 4–5 weeks post-partum (p < 0.001). In contrast, no increase in cortisol in plasma or skim milk was observed in control group (Fig. 2). Milk cortisol concentrations increased significantly (p < 0.001) (from 0.3 ± 0.1 to 2.8 ± 1.1 ng/ml) 8 h after ACTH-Z injection, corresponding to the plasma cortisol response in cows 1–2 weeks post-partum. There was a tendency that adrenocortical response to ACTH-Z was higher 4–5 weeks post-partum than that 1–2 weeks post-partum in cows (Fig. 2). There was a significant correlation between plasma and skim milk cortisol concentrations in ACTH-Z-treated cows 1–2 weeks post-partum (r = 0.77, p < 0.001) and at 4–5 weeks post-partum (r = 0.71, p < 0.003).

**Discussion**

The long-acting ACTH-Z challenge in cows has been reported by Soma et al. (1984) and Miyazawa et al. (1987) who injected 1 mg ACTH-Z. ACTH-Z has also been used for inducing follicular cysts in cattle (Kawate et al. 1996; Ribadu et al. 2000). Kawate et al. (1996) used 3 mg ACTH-Z for 14 days to induce follicular cysts, while Ribadu et al. (2000) were able to induce the cysts after injection of 1 mg ACTH-Z for 7 days. The results of the current study showed that 0.5 mg ACTH-Z was sufficient to examine the adrenocortical response.

It had not been known when plasma cortisol concentrations start to rise significantly after ACTH-Z administration. This study revealed that cows responded to ACTH-Z as early as 30 min after the injection, similar to the response to ACTH. The peak of plasma cortisol concentrations was seen 4 h after the ACTH-Z challenge.

Duration of the elevation in plasma cortisol concentrations after ACTH-Z was approximately 10 h in the current study. This may suggest that response of plasma cortisol after ACTH-Z injection can be monitored by measuring cortisol concentrations before and 8–10 h after the administration. This interval of blood sampling after ACTH-Z injection could be convenient if milk samples were used for cortisol analysis. ACTH-Z can be injected shortly after morning milking, and cortisol can be determined in milk samples collected at the morning milking before ACTH-Z injection and at the afternoon milking.

![Fig. 1. Plasma cortisol concentrations in beef cows after ACTH and ACTH-Z treatment (median and interquartil range, n = 4). There were significant differences in cortisol concentrations by different time after treatment (a. p = 0.002; df 7, b. p = 0.000; df 23, c. p = 0.002; df 7). *Significantly higher than the basal value (p < 0.001). 0 h: immediately before treatment.](image-url)
It was yet to be examined whether the response of the adrenal cortex of cows to ACTH-Z would result in depression of the adrenal function. A rapid ACTH test was conducted in the cows 3 days before and 48 h after the ACTH-Z challenge. The peak plasma cortisol concentrations after 0.25 mg ACTH (equivalent to 25 IU) injection in the beef cows shown in this study were well comparable with the values in dairy cows reported by Yoshida and Nakao (2005). Even after the ACTH-Z challenge, the cows still had a high plasma cortisol response to ACTH. It is, therefore, indicated that the ACTH-Z challenge may not cause suppression of the adrenocortical function. These results suggest that the ACTH-Z challenge can be applied in cows without side effect on the adrenal function.

A number of previous studies showed that cortisol concentrations in milk that are significantly lower than the concentrations in plasma can be used as an indicator of adrenocortical function in cows (Gwazdauskas et al. 1977; Fox et al. 1981; Termeulen et al. 1981; Verkert et al. 1994). Termeulen et al. (1981) reported that the profile of cortisol in plasma and milk during 4 h after ACTH injection were similar and suggested that cortisol concentrations in milk indicate cortisol concentrations in blood.

The ACTH-Z challenge based on cortisol concentrations in milk collected at morning milking before the challenge and at afternoon milking approximately 8 h after the challenge was applied to study adrenocortical function in lactating dairy cows 1–2 weeks and 4–5 weeks post-partum. There was a tendency that cows had a slightly higher adrenocortical response to ACTH-Z 4–5 weeks post-partum than that 1–2 weeks post-partum. This trend corresponds to the changing state of energy balance during a post-partum period, being lowest at approximately 2 weeks post-partum and improving 2 weeks later (Grummer et al. 2010).

The response of plasma cortisol after ACTH-Z corresponded well to skim milk cortisol concentrations. The results obtained in this study suggest that elevated levels of plasma cortisol are maintained for approximately 10 h after ACTH-Z treatment without adverse effect on adrenocortical function and that a long-acting ACTH-Z challenge based on cortisol concentrations in milk, which were collected at the morning and the afternoon milking, can be a useful tool to monitor adrenocortical function in cows.

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**Conflict of interest**

None of the authors have any conflict of interest to declare.

**Author contributions**

NC Thinh and T Nakao assisted in study design, data collection, data analysis and editing manuscript. C Yoshida and ST Long assisted in study design and data collection. M Yusuf assisted in data analysis and editing manuscript.
References

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