**ภาคผนวก**

ประกอบด้วย

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# ภาคผนวก 1

# Changes of body condition score, body weight and some metabolic profiles in relation to times of first ovulatory response and subsequent postpartum ovarian activity in tropical Holstein cows

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## Contents

Forty-eight tropical Holstein (HF ≥ 75%) cows were assessed from 2 weeks prepartum until 10 weeks postpartum (pp). Serum progesterone (P4) was assayed 2 times a week for detection of the first ovulation pp and subsequent ovarian activity. Pre-calving non-esterified fatty acids (NEFA) were analysed once weekly and the post-calving beta-hydroxybutyrate (BHB) were quantified at 1, 2, 3, 5 and 7 weeks pp. Group I (ER) - 9 cows (18.8%) had the first ovulatory response ≤ 30 days pp with subsequent normal ovarian cyclicity, Group II (ER+AB) - 16 cows (33.3%) had the first ovulatory response ≤ 30 days pp and Group III (LR+AB) - 23 cows (47.9%) had the first ovulatory response > 30 days pp – both Groups II and III showed subsequent abnormal ovarian cyclicity. No significant differences of the mean BCS, BW and pre-calving NEFA between groups were observed but only the mean BCS and BW was significantly different between pre- and postpartum periods ($P < 0.001$). While, the mean levels of BHB in Groups II (ER+AB) and III (LR+AB) were moderately high at 2, 3 and 5 weeks pp but no significant differences were detected between groups and weeks pp.

Nevertheless, a number of cows with high values of BHB pp (> 1.4 mmol/L) tended to be larger in Groups II (ER+AB) and III (LR+AB), compared with Group I (ER) (P = 0.06). In conclusion, a high number of tropical Holstein cows were seriously affected by early or late first ovulatory response pp with subsequent abnormal ovarian activity. Furthermore, the present results suggested that the affected cows were principally related to the negative energy balance during pp period.

## Introduction

Several previous studies indicated a closed-link between the negative energy balance (NEB) and reproductive performance in lactating dairy cows (Reksen et al. 2002; Kim and Suh 2003; Shrestha et al. 2005; Konigsson et al. 2008). The energy demand of cows increases during the last weeks of gestation, while the fetus is growing larger and the energy need of the cows during early postpartum period is even greater for milk production. Holter et al. (1997) reported that there was a significant negative correlation between temperature and humidity index (THI), an indicator of heat-stress condition and dry matter intake (DMI) for cows and a higher state of negative energy balance was recorded in postpartum cows (West et al. 2003). A substantial proportion of dairy cows under NEB also revealed a stage of delayed first ovulation and subsequent ovarian disorders (Opsomer et al. 2000; Hommeida et al. 2005; Kornmatitsuk et al. 2008). A decrease of plasma insulin, glucose and IGF-I levels, which required for normal development of the follicle was also indicated in cows with negative energy balance (Konigsson et al. 2008; Rotz 2008).

Body condition score (BCS) and body weight (BW) change is apparently related to the nutritional status of pre- and postpartum dairy cows. It is recommended that the cow's

BCS should not decrease ≥ 1 unit within a month postpartum. Cows with greater loss of body condition score and body weight during pre- and post-calving periods had a greater risk of having a delay of first ovulation and abnormal ovarian activity postpartum (Kim & Suh, 2003; Shrestha et al. 2005). In addition, Hoedemaker et al. (2009) indicated that cows with no BCS loss pre-calving were more likely to be cycling at 3 and 5 weeks postpartum.

Monitoring of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) was purposed as a tool for nutritional management in a herd level (Melendez & Risco 2005). NEFA in blood are derived from lipid stores and used as an alternative source of energy. However, limited amount of NEFA can be oxidized to completion by the tricarboxylic acid cycle of the liver or be exported from the liver as very low density lipoproteins. In the case of excessive fat mobilization, associated with marked formation of acetyl-coenzyme A, the tricarboxylic acid cycle cannot fully metabolized fatty acids. As a consequence, acetyl-coenzyme A is converted to acetoacetate, which is then reduce to beta-hydroxybutyrate (BHB) or spontaneously decarboxylized to acetone. Therefore, measurement of NEFA and BHB could serve as a reliable indicator of lipid mobilization and energy status in dairy cows (Reist et al. 2000). Leblanc (2010) mentioned that high NEFA levels (> 0.4 mmol/L) near term and serum BHB > 1.2 to 1.4 mmol/L in the first or second week postpartum was associated with increased risk of periparturient diseases, prolonged postpartum anovulation and higher culling rate.

The first objective of this study was to investigate changes of body condition scores, body weight, pre-calving NEFA and post-calving BHB in relation to postpartum ovarian activity of tropical Holstein cows. The second objective was to evaluate the possible

involvement of pre- or post-calving negative energy balance on postpartum ovarian disorders.

## Materials and Methods

### Animals and experimental designs

Forty-eight Holstein-Friesian (HF ≥ 75%) lactating cows, parity 1 – 4 were randomly selected for the present study. The cows were kept in the research farm of the Dairy Promotion Organization of Thailand (D.P.O.), located in Saraburi province, in a central region of Thailand. The area has a tropical climate with three distinct seasons; summer (Mar – Jun), rainy (Jul – Oct) and winter (Nov – Feb). The mean temperature and humidity index (THI), calculated according to NOAA (1976) varied between 72 – 79 units (the Climate Information Services, Thai Meteorological Department).

Cows were fed 2 times per day to meet their maintenance, growth and production requirements, according to NRC (2001). The main feed offered was grass silage and concentrate. Water was given *ad libitum*. Cows were machine-milked twice daily (0700 and 1500 h) and the average 305-day milk production was 4,000 – 5,000 kg.

All procedures of the experimental protocol were approved by the local animal ethics committee, Mahidol University as guided by the National Research Council, Bangkok, Thailand.

### Body condition score and body weight

Body condition scores (BCS) was determined visually according to Edmonson et al. (1989) using a five-point scale with 0.25 increments. The evaluation of BCS was always performed by the same person. Cows were scored weekly from 2 weeks prior to

parturition until 10 weeks postpartum. Body weight was measured by using a heart girth measuring tape concurrent with assessment of BCS.

**Blood collection**

Blood samples were collected from the coccygeal vein in 6-ml plain vacuumed tubes (BD Vacutainer®, Becton Dickinson and Company, NJ, USA) with 21-gauge needle and 1-inch long. After leaving for 1 h at 4°C, blood samples were centrifuged at 1000 × *g* for 15 min and serum samples were harvested and kept at -20°C for analyses of serum progesterone (P4), non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB).

**Progesterone analyses**

Analyses of progesterone (P4) levels were determined 2 times a week (Tuesday and Friday) from calving to 10 weeks postpartum. The analyses were performed by enzyme immunoassay according to Munro and Stabenfeldt (1984) and Kornmatitsuk et al. (2007). The intra- and inter-assay coefficients of variation (based on 2 control samples) were 8.0 and 14.0%, respectively.

P4 data was used to determine the day of first ovulation and subsequent postpartum ovarian activity.

**Determination of non-esterified fatty acids**

Serum levels of non-esterified fatty acids (NEFA) were analysed at 1 (2 – 8 days) and 2 (9 – 15 days) weeks pre-calving by enzymatic-colorimetric methods (Randox NEFA®, Randox Laboratories Ltd., United Kingdom). The assay procedure was performed according to the manufacture's guideline. The linearity of the standard curve was determined with a range of standards: 0.1, 0.5, 1.0 and 1.91 mmol/L. The correlation coefficient ($r^2$) of the standard curve was 0.99.

**Determination of beta-hydroxybutyrate**

Serum levels of beta-hydroxybutyrate (BHB) were determined at 1, 2, 3, 5 and 7 weeks postpartum by kinetic enzymatic method with commercially available kits (Ranbut® D-3-hydroxybutyrate, Randox Laboratories Ltd., United Kingdom). Lower detection limit of the kits was 0.1 mmol/L.

**Evaluation of reproductive performance**

The cow's reproductive performance was subsequently evaluated based on the artificial insemination (AI) records. All cows were subjected to the synchronization protocol of ovulation, using GnRH (Fertagyl®, Intervet International B.V., Boxmeer, Netherlands), an intravaginal progesterone release device (Eazi Breed® CIDR, InterAgri, Hamilton, New Zealand) and PGF2alpha (Lutalyse®, Pharmacia, Puurs, Belgium) or Ovsynch + CIDR protocol at day 70 postpartum.

**Statistical analyses**

Descriptive data was presented as the mean and the standard errors of the mean (mean ± SEM) and frequency tables. Statistical analyses were carried out by using a commercial statistical package (SPSS® version 16, SPSS Inc., IL, USA). The Procedure Mixed between-within subjects analysis of variance was used and differences of the means were obtained by Tukey test and/or Dunnett's test. Categorical variables were analysed by Fisher's exact test and Chi-square test. Probability values of less than 0.05 were considered to be significant.

## Results

Based on individual serum P4 profiles, the cows could be categorised into 3 groups; Group I (ER), N = 9/48 (18.8%) – cows showed early response with the first ovulation occurred ≤ 30 days postpartum and following with normal ovarian cyclicity, Group II

(ER+AB), N = 16/48 (33.3%) – cows showed early response with the first ovulation occurred ≤ 30 days postpartum and following with abnormal ovarian cyclicity and Group III (LR+AB), N = 23/48 (47.9%) – cows showed late response with the first ovulation occurred > 30 days postpartum and following with abnormal ovarian cyclicity. The patterns of abnormal ovarian cyclicity in Group II (ER+AB) consisted of; 1) persistent CL, 2) cessation of ovarian activity and 3) short luteal phase, while the most observed patterns in Group III (LR+AB) were; 1) delayed ovulation and 2) anovulation. Details of clinical data and reproductive performance are presented in Table 1.

**Changes of body condition score (BCS)**

The pre-calving means of BCS in Groups I (ER), II (ER+AB) and III (LR+AB) were 3.18 ± 0.09, 3.26 ± 0.09 and 3.23 ± 0.05, respectively. After calving, the mean BCS of all groups started decreasing sharply during the first week postpartum and reached a constant level during 3 – 10 weeks postpartum. The post-calving means of BCS in Groups I (ER), II (ER+AB) and III (LR+AB) at the end of studying period were 2.57 ± 0.07, 2.40 ± 0.05 and 2.41 ± 0.06, respectively (Fig. 1).

No difference of the mean BCS was found between groups ($P > 0.05$) but it was significant by weeks between pre- and post-calving periods ($P < 0.001$).

**Changes of body weight (BW)**

Changes of the mean BW had a similar pattern, according to changes of the mean BCS during pre- and post-calving periods. The pre-calving means of BW in Groups I (ER), II (ER+AB) and III (LR+AB) were 540.0 ± 28.2, 535.8 ± 14.9 and 541.0 ± 12.5 kg, respectively. A continuing decline of BW was observed from 1 – 2 weeks postpartum until the end of studying period and the means of BW in Groups I (ER), II (ER+AB) and III (LR+AB) were 452.8 ± 21.8, 449.5 ± 15.8 and 442.6 ± 11.3, respectively (Fig. 2).

No difference of the mean BW was found between groups ($P > 0.05$) but it was significant different by weeks between pre- and post-calving periods ($P < 0.001$).

**Pre-calving changes of non-esterified fatty acids (NEFA)**

The pre-calving mean NEFA levels of all groups maintained at about 0.3 – 0.4 mmol/L. The mean NEFA levels at 1 and 2 weeks before parturition in Groups I (ER), II (ER+AB) and III (LR+AB) were 0.38 ± 0.04, 0.42 ± 0.12; 0.38 ± 0.04, 0.42 ± 0.12 and 0.38 ± 0.04, 0.42 ± 0.12, respectively (Fig. 3). No significant differences between groups and weeks postpartum were recorded ($P > 0.05$).

In addition, the number of cows with high levels of pre-calving NEFA (a cut-off level = 0.4 mmol/L) was not different between groups, as presented in Table 2 ($P > 0.05$).

**Post-calving changes of beta-hydroxybutyrate (BHB)**

The post-calving changes of BHB levels during the first week postpartum were similar among groups and the levels in Groups I (ER), II (ER+AB) and III (LR+AB) were equal to 0.55 ± 0.12, 0.59 ± 0.08 and 0.58 ± 0.10, respectively. Afterwards, the mean BHB levels in Groups II (ER+AB) and III (LR+AB) were moderately higher at 2, 3 and 5 weeks postpartum, compared with Group I (ER) (Fig. 3) but the post-calving means of BHB were not significant different between groups and weeks postpartum ($P > 0.05$). Nevertheless, the number of cows with high values of BHB postpartum (a cut-off level = 1.4 mmol/L) tended to be larger in Groups II (ER+AB) and III (LR+AB), compared with Group I (ER) as showed in Table 3 ($P = 0.06$).

## Discussion

In the present results, about 33% of cows (Group II, ER+AB) showed early response with the first ovulation occurred ≤ 30 days postpartum and following with abnormal

ovarian cyclicity and 48% of cows (Group III, LR+AB) showed late response with the first ovulation occurred > 30 days postpartum and following with abnormal ovarian cyclicity. The present number of affected cows was similar to our previous report, in which about 30% of cows affected by heat stress resumed normal ovarian function postpartum (Kornmatitsuk et al. 2008). However, Reist et al. (2000) and Reksen et al. (2002) reported that about 50% of cows had normal ovarian function postpartum, whether, those papers implied the cows with late first ovulatory response of a 24-d or 30-d postpartum. Thus, it was clearly shown that a rather high number of tropical Holstein cows were suffering from postpartum ovarian disorders.

Loss of BCS near term of pregnant cows resulted in the increased occurrence of metabolic and reproductive diseases and a longer interval to first breeding postpartum (Kim & Suh, 2003). Hoedemaker et al. (2009) also indicated that body condition loss during prepartum period affected BCS status at parturition and during lactation which was linked to a higher incidence of subsequent reproductive disorders, a higher culling rate and lower fertility. However, there was no evidence in the present study that cows were losing their BCS and BW during the last 2 weeks before calving. This was in accordance with the present results of NEFA analyses, which had showed no significant differences between groups and weeks prepartum. Therefore, cows in Groups II (ER+AB) and III (LR+AB), with early or late ovulatory responses and abnormal ovarian cyclicity were not directly related to NEB during pre-calving period.

A sharp decline of BCS and BW in all groups was observed during the first and second weeks postpartum and maintained throughout the study. It was not showed in the present results that both parameters could be used to indicate differences of the nutritional status among cows in different groups. This might be caused by different

genetics and non-genetics factors affected on Holstein cows in tropical climate, in which the dry matter intake of cows is generally low (Holter et al. 1997; West et al. 2003). There was also an increase of BHB levels at 2, 3 and 5 weeks postpartum, particularly a higher release of BHB was observed in Groups II (ER+AB) and III (LR+AB), compared with Group I (ER). In addition, a number of cows in Groups II (ER+AB) and III (LR+AB) with the post-calving rise of BHB over a cut-off level of 1.4 mmol/L tended to be larger, compared with Group I (ER). These all data indicated that cows in Groups II (ER+AB) and III (LR+AB) might have poorer nutritional status and/or deeper negative energy balance. It was in accordance with previous reports from several investigators, showed that cows with high BHB > 1.2 to 1.4 mmol/L during early postpartum period was associated with increased risk of periparturient diseases, prolonged postpartum anovulation and higher culling rate (Kafi & Mirzaei 2010; LeBlanc 2010).

In conclusion, a high number of tropical Holstein cows were affected by postpartum ovarian dysfunction and cows with abnormal ovarian cyclicity in the present study were not directly caused by pre-calving NEB, whereas it was indicated that the affected cows were principally related to the negative energy balance during postpartum period.

## Acknowledgement

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Table 1. Clinical data and the reproductive performance in Groups I (ER), II (ER+AB) and III (LR+AB)

| Parameter | Animal groups | | | Total |
|---|---|---|---|---|
| | Group I (ER) | Group II (ER+AB) | Group III (LR+AB) | |
| No. of cows | 9 (18.8%) | 16 (33.3%) | 23 (47.9%) | 48 (100%) |
| Day of first ovulation* | 22.4 (±1.2) | 20.6 (±0.9) | 47 (±2.2) | 33.6 (±2.2) |
| Pregnancy at 180 days postpartum | 5/9 (55.6%) | 7/16 (43.8%) | 7/23 (30.4%) | 19/48 (39.6%) |
| Days open | 177 (±51.8) | 223.8 (±48.2) | 197.7 (±37.1) | 201.9 (±25.1) |
| No. of culling | 0/9 (0%) | 3/16 (18.8%) | 4/23 (17.4%) | 7/48 (14.6%) |

*$P < 0.001$

Table 2. Distribution of cows with the pre-calving levels of non-esterified fatty acids (NEFA) over a cut-off level (0.4 mmol/L) in Groups I (ER), II (ER+AB) and III (LR+AB)

| NEFA (mmol/L) | Animal groups* | | | Total |
|---|---|---|---|---|
| | Group I (ER) | Group II (ER+AB) | Group III (LR+AB) | |
| > 0.4 | 3 (33.3%) | 6 (37.5%) | 6 (26.0%) | 15 (31.3%) |
| ≤ 0.4 | 6 (66.7%) | 10 (62.5%) | 17 (73.9%) | 33 (68.7%) |
| Total | 9 (100%) | 16 (100%) | 23 (100%) | 48 (100%) |

*P > 0.05

Table 3. Distribution of cows with the post-calving levels of beta-hydroxybutyrate (BHB) over a cut-off level (1.4 mmol/L) in Groups I (ER), II (ER+AB) and III (LR+AB)

| BHB (mmol/L) | Animal groups* | | | Total |
|---|---|---|---|---|
| | Group I (ER) | Group II (ER+AB) | Group III (LR+AB) | |
| > 1.4 | 1 (11.1%) | 9 (56.3%) | 7 (30.5%) | 17 (35.4%) |
| ≤ 1.4 | 8 (88.9%) | 7 (43.7%) | 16 (69.5%) | 31 (64.6%) |
| Total | 9 (100%) | 16 (100%) | 23 (100%) | 48 (100%) |

*P = 0.06

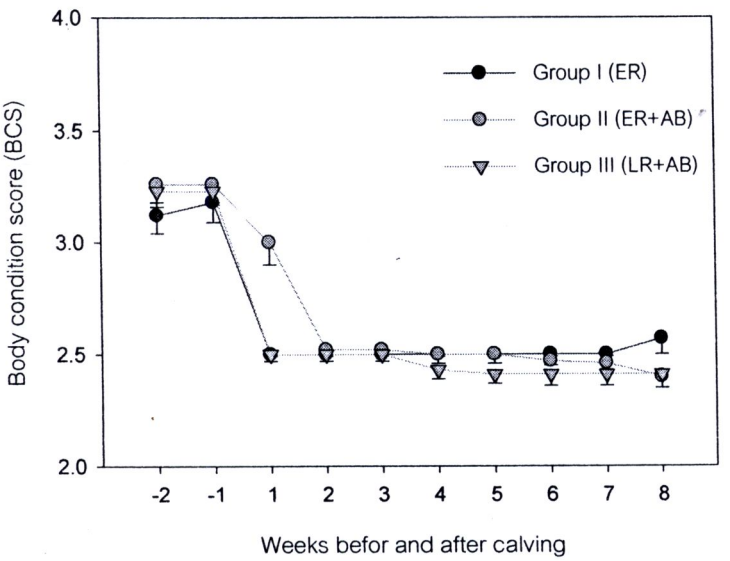
## Legends of figures

Fig. 1. Changes of the mean body condition score (BCS) before and after calving in Groups I (ER), II (ER+AB) and III (LR+AB)

Fig. 2. Changes of the mean body weight (BW) before and after calving in Groups I (ER), II (ER+AB) and III (LR+AB)

Fig. 3. Changes of the pre-calving mean non-esterified fatty acids (NEFA) and the post-calving mean beta-hydroxybutyrate (BHB) in Groups I (ER), II (ER+AB) and III (LR+AB)

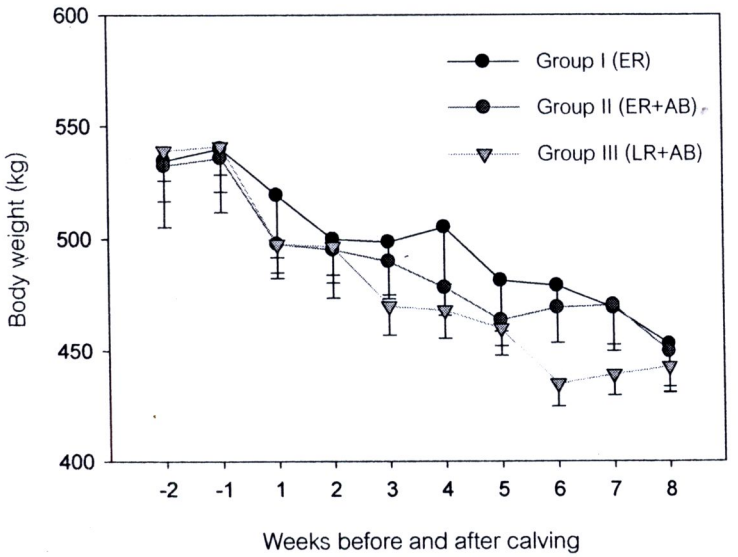
1


4.0
3.5
3.0
2.5
2.0
Body condition score (BCS)
Group I (ER)
Group II (ER+AB)
Group III (LR+AB)
-2
-1
1
2
3
4
5
6
7
8
Weeks befor and after calving

2

3

1


600
550
500
450
400
Body weight (kg)
Group I (ER)
Group II (ER+AB)
Group III (LR+AB)
-2
-1
1
2
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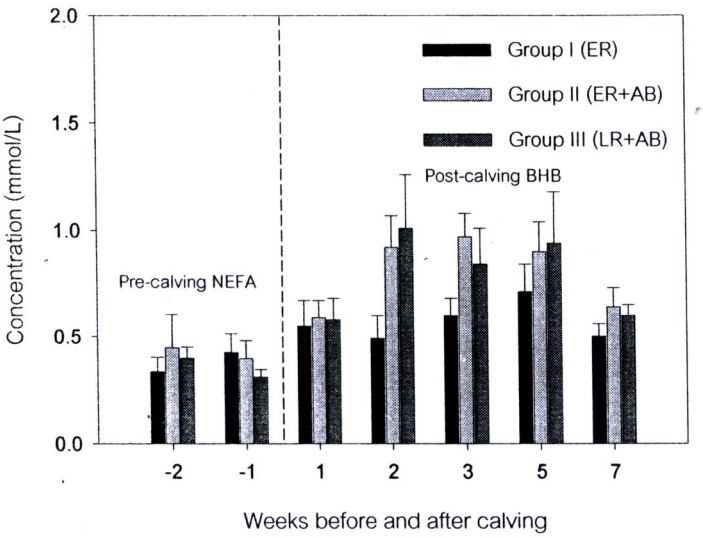
4
5
6
7
8
Weeks before and after calving

2

3

1


2.0
1.5
1.0
0.5
0.0
Concentration (mmol/L)
Group I (ER)
Group II (ER+AB)
Group III (LR+AB)
Post-calving BHB
Pre-calving NEFA
-2
-1
1
2
3
5
7
Weeks before and after calving

2

3

# ภาคผนวก 2

# Determination of non-esterified fatty acids in bovine serum: evaluation of a modified Randox NEFA® kit with reduced sample and reagent volumes

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## Abstract

The present study was aimed to determine non-esterified fatty acids (NEFA) in bovine serum, using the commercial Randox NEFA® kit with reduced sample and reagent volumes. In the original NEFA-assay (1:1) protocol, the linearity test of standard curve with 4- point concentrations; 0.1, 0.5, 1.0 and 1.91 mmol/L resulted in the coefficient of determination ($r^2$) of 0.9995, while, the values of the modified NEFA-assay protocols of 1:2, 1:4 and 1:5 were 0.9939, 0.9956 and 0.9896, respectively. The slopes and intercepts did not differ among the protocols. In the precision analysis, mean values (± SD) of standard (1.0 mmol/L), measured by the original (1:1) and modified NEFA-assay (1:5) protocols were 1.02 ± 0.03 and 1.03 ± 0.02 mmol/L, respectively. For Bland and Altman statistical analysis of the protocol agreement, an average of 30 sets of differences (bias) between 2 protocols was calculated to be 0.03 mmol/L with SD of 0.05 mmol/L and the 95% confidence interval of 0.01 - 0.04 mmol/L. In conclusion, the analysis of serum NEFA levels in bovine serum by means of the modified Randox NEFA® kit with a 5-time reduction (1:5) of sample and reagent volumes demonstrated a comparable result to the original NEFA-assay protocol.

**Key words:** modified assay, non-esterified fatty acids, reduction of sample and reagent volumes, postpartum cows

## Introduction

Measurement of non-esterified fatty acids (NEFA) has been purposed as a tool for monitoring of nutritional status in transitional cows, in which the physiological status is remarkably changed such as rapid fetal development, parturition and start of milk production (Garrett, 2004). An increase of NEFA indirectly indicates a degree of negative energy balance, which is mostly associated with lower subsequent reproductive performance in lactating cows, determined by lower artificial insemination (AI) conception rates and longer days open (Westwood *et al.*, 2002).

Blood circulating NEFA, derived from fat mobilization are taken up by the udder for producing milk fat in a form of milk triglycerides or are oxidized in the liver as an alternative energy source (Konigsson *et al.*, 2008). In the liver, NEFA are oxidized to form acetyl CoA before introducing into the tricarboxylic acid cycle or can be used for the synthesis of cholesterol and ketone bodies. Some of NEFA are esterified to triacylglycerol (TAG) before secreting to the blood stream as very low density lipoprotein (VLDL) particles. However, the capability of ruminant liver for VLDL export is physiologically limited and the accumulation of TAG is resulted in hepatic lipidosis or fatty liver (Bobe *et al.*, 2004; Grummer, 2008).

Several studies have demonstrated the relationship between high NEFA or fatty liver and development of retained placenta, ketosis, metritis and left displaced abomasum (Rukkwamsuk *et al.*, 1999; Westwood *et al.*, 2002; Bobe *et al.*, 2004; Seifi *et al.*, 2007). Furthermore, a cow with a higher NEFA level (~ 0.4 mmol/L) has a higher chance of anovulation during the first follicular wave postpartum (Kawashima *et al.*, 2007). This was reported as an effect of NEFA on suboptimal ovarian micro-environment from decreased gluconeogenesis in the liver, causing sub-fertility and infertility in lactating cows (Bobe *et al.*, 2004).

There are various methods for analysis of NEFA levels in various samples such as Thin-layer Chromatography, Gas-liquid Chromatography, High Performance Liquid Chromatography (HPLC), Capillary Zone Electrophoresis (CE), Solid-phase microextraction (SPE) and Gas Chromatography-Mass Spectrometry (GC/MS) (Miksa *et al.*, 2004). However, the enzymatic methods for determining of serum NEFA levels are more practical and ready to be used in most laboratories and the results are also very well accepted by the practitioners (Westwood *et al.*, 2002; Konigsson *et al.*, 2008). There are several available NEFA kits on the market. However, a total cost per sample unit of these commercial products is quite high, particulary for the small-holder dairy farms in Thailand and in turn, leading to a limitation of NEFA use for monitoring of metabolic disturbances in transitional cows.

Thus, in order to decrease assay-unit cost and increase a wide application of NEFA analysis, the modified-assay protocols of Randox NEFA® kit by a reduction of sample sizes and reagent volumes were evaluated.

## Materials and Methods

### Experimental design

Different standard curves were performed by use of reduced volumes of standards and reagents for 2 (1:2), 4 (1:4) and 5 times (1:5), respectively. The optimum reduction protocol was verified in a comparison with the original NEFA-assay protocol for the precision by use of the standard (1.0 mmol/L) (N =10) and assay-control samples (Randox Assayed Multi-sera, Level 3, N = 5). An appropriate volume-reduction NEFA protocol was selected for further analysis of the protocol agreement, in which a total of 30 sets of differences were observed.

### Blood collection

Blood samples were obtained from 6 purebred Holstein Friesian cows, parities 1-5 during 0-7 weeks postpartum. The coccygeal venipuncture was done by using 20-G, 1' length needle with a 6-ml plain vacuum tube (SSTTM II Advance, BD Vacutainer®, Becton Dickinson, Plymouth, UK). The sample was rested at 4°C for 1 hour and then, centrifuged at 1,000 x *g* for 15 minutes. Serum was harvested and stored in the 1.8 ml-microcentrifuge tube at -20°C until further NEFA analysis.

### Preparation of standards and reagents

Standard stock solution (1.0 mmol/L) of non-esterified oleic acid provided by the Randox NEFA® kit (Randox NEFA®, Randox Laboratories Ltd., Crumlin, Co. Antrim, UK) was used to prepare 0.1, 0.5, 1.0 and 1.91 mmol/L working standards, respectively.

The reagent preparations, solution R1 (Acetyl Coenzyme A Synthetase, Ascorbate oxidase, Coenzyme A, adenosine phosphatase [ATP] and 4-aminoantiperine) and solution R2 (Acetyl Coenzyme A Oxidase and Peroxidase), were performed according to the product direction.

### Comparison of the standard curves

In the original protocol (1:1), 4 different working standards; 0.1, 0.5, 1.0 and 1.91 mmol/L, were used. The assay procedure was carried out as described in the NEFA-assay procedures. The same steps were also applied for the modified-assay (1:2, 1:4 and 1:5) protocol with a reduced amount of each working standards and reagents. In the final step, a standard dose-response curve was fitted by plotting the absorbance values against the actual amount of working standard concentrations.

### NEFA-assay procedures

In the original protocol (1:1), 50 l of standards or serum samples were pipetted into 12 x 75 mm. glass-tubes. Thereafter, 1 ml of the solution R1 was added to all tubes. The mixtures were vortexed for 5-10 seconds and incubated at 37°C for 10 minutes. This was followed by adding 2 ml of solution R2,

the tube was mixed and re-incubated at 37°C. After 10 minutes, the mixture was measured for the optical density (OD) at 550 nm.

In the modified-assay protocols (1:5), the amount of standards, serum samples, solution R1 and solution R2 were all reduced by 5 times. In brief, 10 µl of standard stock solution or serum samples, 200 µl of solution R1 and 400 µl of the solution R2 were used, while, steps of the assay protocol was followed as in the original protocol.

In addition, all the standards, serum samples and assay-control (Randox Assayed Multi-sera, Level 3) in both procedures were analysed in duplicate. The calculation of sample NEFA concentration was fitted by using the following equation; mmol/L = Absorbance of sample/Absorbance of standard x Standard concentration (1.0 mmol/L).

**Spectrophotometer conditions**

All UV-visible spectrophotometer detections were conducted at room temperature (25°C) using a double-beam, dual matched silicon photodiode spectrophotometer (Evolution 300 LC®, Thermo Electron Corporation, Cambridge, UK). Semi-micro cuvettes with 1-cm light path were used for all the measurements. A baseline spectrum (blank sample) was performed each day before sample analysis. The instrument was set to operate at a single wavelength of 550 nm.

**Statistical analyses**

Data are presented as mean ± standard deviation (SD). The standard curve was fitted by use of the linear regression analysis, in which the slope and intercept between the curves were compared, using Graphpad Prism version 4.0 (GraphPad Software Inc., CA, USA). The Kolmogorov-Smirnov test was used for the normal distribution analysis and mean values between groups were compared using *t*-test for unpaired data. An agreement assessment of the original (1:1) and modified (1:5) NEFA-assay protocols was performed according to the graphical technique of Bland and Altman analysis (Altman, 1991). Probability values of lesser than 0.05 ($P < 0.05$) were considered to be significant.

## Results

**Linearity of the standard curves**

In the original NEFA-assay protocol, the linearity of the standard curve was determined with a range of standards: 0.1, 0.5, 1.0 and 1.91 mmol/L. A linear regression analysis resulted in a linear curve with the coefficient of determination ($r^2$) of 0.9995. In the modified NEFA-assay protocols, a linear curve could be obtained for each protocol; 1:2, 1:4 and 1:5 with the coefficient of determinations of 0.9939, 0.9956 and 0.9896, respectively. All standard curves were shown as a straight line up to 1.91 mmol/L (Fig1.). In addition, the slopes and intercepts among the protocols were not significantly different ($P = 0.68$).

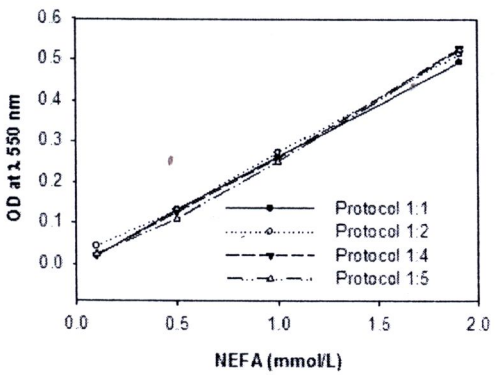


**Fig. 1.** Standard curve of the original (1:1) and modified NEFA-assay (1:2, 1:4 and 1:5) protocols

**Precision of the original (1:1) and modified NEFA-assay (1:5) protocols**

The precision of each protocol was determined on the basis of 10 replicates of 1.0 mmol/L standard solution. Mean values (± SD) of the standard, measured by the original (1:1) and modified NEFA-assay (1:5) protocols were 1.02 ± 0.03 and 1.03 ± 0.02 mmol/L, respectively. There were no significant differences of the mean in both protocols ($P$ = 0.38). The optical density values and the corresponding concentrations for all determinations, measured by the original (1:1) and modified NEFA-assay protocols (1:5) are summarized in Table 1. In addition, the replicated analysis of assay-control of both protocols were also lied within a range of 0.34 - 0.46 mmol/L, as indicated by the kit.

**Table 1.** The optical density values and the corresponding concentrations of the standard (1.0 mmol/L), measured by the original (1:1) and modified NEFA-assay (1:5) protocols

| Sample | OD 1:1 | NEFA 1:1 (mmol/L) | OD 1:5 | NEFA 1:5 (mmol/L) |
|---|---|---|---|---|
| 1 | 0.2450 | 0.99 | 0.2615 | 1.04 |
| 2 | 0.2471 | 1.00 | 0.2607 | 1.03 |
| 3 | 0.2548 | 1.03 | 0.2561 | 1.01 |
| 4 | 0.2564 | 1.03 | 0.2580 | 1.02 |
| 5 | 0.2636 | 1.06 | 0.2611 | 1.03 |
| 6 | 0.2666 | 1.07 | 0.2675 | 1.06 |
| 7 | 0.2556 | 1.03 | 0.2562 | 1.02 |
| 8 | 0.2439 | 0.98 | 0.2588 | 1.03 |
| 9 | 0.2450 | 0.99 | 0.2499 | 0.99 |
| 10 | 0.2482 | 1.00 | 0.2656 | 1.05 |
| Average | 0.2526 | 1.02 | 0.2595 | 1.03 |
| SD | 0.0081 | 0.03 | 0.0050 | 0.02 |

**Assessment on the protocol agreement**

According to graphical technique of Bland and Altman for the protocol agreement calculation, an average of 30 sets of differences (bias) between 2 protocols (1:1 and 1:5 NEFA-assay protocols) was calculated to be 0.03 mmol/L with SD of 0.05 mmol/L. The standard error of the mean (SEM) was determined to be 0.01 mmol/L and the 95% confidence interval for the bias, using the formula [mean $\pm$ 2SEM), resulted in a range of 0.01 - 0.04 mmol/L.

## Discussion

The availability of a commercial test kit for measuring NEFA concentration in serum or plasma makes its quantitation relatively straightforward. However, the original NEFA-assay protocol with recommended sample and reagent volumes is leading to a high cost per sample unit. This also results in a decrease of demand and benefits for its use on monitoring of nutritional status in transitional cows and feeding management protocol, particulary for the small-holder dairy farm in Thailand.

In the present study, the linearity of all standard curves, determined by the coefficient of determination ($r^2$) was rather high and straight up to the maximum NEFA concentration (1.91 mmol/L). In the original NEFA-assay protocol, the $r^2$ value was 0.9995. In the modified NEFA-assay (1:2. 1:4 and 1:5) protocols, the values varied between 0.9896 - 0.9956. Although, the lowest $r^2$ value was observed in the modified NEFA-assay (1:5) protocol, the slope and intercept among the protocols were not statistically different. It was indicated that all modified NEFA-assay had a feasibility to be used for determination of NEFA levels in bovine serum.

To further test the precision of the modified NEFA-assay (1:5) protocol to the original NEFA-assay protocol, the standard of 1.0 mmol/L was used to evaluate. No significant differences of the mean in both protocols were observed. In the replicated analysis of assay-control in both protocols, the values were also lied within a recommended range by the kits. In the graphical technique of Bland and Altman for agreement calculation, the mean of 30 sets of differences between 2 protocols was 0.03 mmol/L with SD of 0.05 mmol/L and the 95% confidence interval for the bias, using the formula [mean $\pm$ 2SEM], resulted in a range of 0.01 - 0.04 mmol/L. Thus, the present results illustrated that the original NEFA-assay protocol (1:1) could be substituted by the modified NEFA-assay protocol (1:5).

In the literatures, a higher increase of NEFA level > 0.4 mmol/L was related to a condition of negative energy balance (NEB) in lactating cows (Garrett, 2004). The NEB during postpartum period was also highly correlated with the days to first ovulation (Bossaert *et al.*, 2008; Stenglrde *et al.*, 2008; Tanaka *et al.*, 2008) and anovulation during the first follicular wave postpartum (Kawashima *et al.*, 2007). Thus, the NEFA measurement is recommended to be used for monitoring of the wellness of nutritional management in dairy farm (Stenglrde *et al.*, 2008; Tanaka *et al.*, 2008).

In conclusion, the modified Randox NEFA® kit with a 5-time reduction of sample and reagent volumes demonstrated a comparable result to the original NEFA-assay protocol and could possibly be substituted for detection and quantitation of NEFA concentration in bovine serum.

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# การวิเคราะห์กรดไขมันชนิด non-esterified ในซีรั่มโค: การประเมินผลการดัดแปลงชุดทดสอบ Randox NEFA® ที่มีการลดปริมาณตัวอย่างและสารทำปฏิกิริยา

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## บทคัดย่อ

การศึกษาครั้งนี้มีจุดประสงค์เพื่อตรวจวิเคราะห์ระดับความเข้มข้นของกรดไขมันชนิด non-esterified (NEFA) ในซีรั่มโคด้วยชุดทดสอบ Randox NEFA® ที่มีการลดปริมาณตัวอย่างและสารทำปฏิกิริยาลง จากผลการศึกษา พบว่าความสัมพันธ์เชิงเส้นตรงของกราฟมาตรฐานที่ความเข้มข้น 4 ระดับ ได้แก่ 0.1, 0.5, 1.0 และ1.91 มิลลิโมล/ลิตรในวิธีการตรวจวิเคราะห์แบบเดิมที่กำหนดโดยชุดทดสอบ (1:1) นั้นให้ค่าสัมประสิทธิ์สหสัมพันธ์ ($r^2$) เท่ากับ 0.9995 เปรียบเทียบกับวิธีการตรวจวิเคราะห์ที่มีการดัดแปลงที่ลดปริมาณตัวอย่างและสารทำปฏิกิริยาลง 2 เท่า (1:2), 4 เท่า (1:4) และ 5 เท่า (1:5) นั้นให้ค่าเท่ากับ 0.9939, 0.9956 และ 0.9896 ตามลำดับ ขณะที่ค่าความชัน (slope) และจุดตัด (intercept) ของกราฟมาตรฐานระหว่างทุกวิธีการตรวจวิเคราะห์นั้นไม่มีความแตกต่างทางสถิติ และเมื่อทดสอบหาความแม่นยำเปรียบเทียบระหว่างวิธีการตรวจวิเคราะห์แบบเดิม และแบบดัดแปลง 1:5 โดยใช้สารมาตรฐานที่ 1.0 มิลลิโมล/ลิตรนั้น ให้ค่าความเข้มข้นที่วัดได้เท่ากับ $1.02 \pm 0.03$ และ $1.03 \pm 0.02$ มิลลิโมล/ลิตร ตามลำดับ สำหรับการวิเคราะห์ทางสถิติโดยวิธีของ Bland และ Altman เปรียบเทียบระหว่างวิธีการตรวจวิเคราะห์ทั้งสองในซีรั่มโคจำนวน 30 ตัวอย่าง พบว่าค่าเฉลี่ยความแตกต่างระหว่างระดับความเข้มข้นของ NEFA เท่ากับ 0.03 มิลลิโมล/ลิตร และมีค่าความแปรปรวนเท่ากับ 0.05 มิลลิโมล/ลิตร โดยช่วง 95% ของความแตกต่างระหว่างระดับความเข้มข้นของ NEFA มีค่าระหว่าง 0.01 - 0.04 มิลลิโมล/ลิตร ดังนั้นจากการศึกษาครั้งนี้ สรุปได้ว่าการตรวจวิเคราะห์ NEFA ในซีรั่มโคโดยลดปริมาณตัวอย่างและสารทำปฏิกิริยาลง 5 เท่า (1:5) ให้ผลการตรวจวิเคราะห์ระดับความเข้มข้นของ NEFA ไม่แตกต่างจากวิธีที่กำหนดโดยชุดทดสอบ

คำสำคัญ: การดัดแปลงวิธีการตรวจวิเคราะห์ กรดไขมันชนิด non-esterified การลดปริมาณตัวอย่างและสารทำปฏิกิริยา แม่โคหลังคลอด