Technical note: Comparison of 3 methods for analyzing areas under the curve for glucose and nonesterified fatty acids concentrations following epinephrine challenge in dairy cows

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ABSTRACT

The objective of the study was to compare 3 methods for calculating the area under the curve (AUC) for plasma glucose and nonesterified fatty acids (NEFA) after an intravenous epinephrine (EPI) challenge in dairy cows. Cows were assigned to 1 of 6 dietary niacin treatments in a completely randomized 6×6 Latin square with an extra period to measure carryover effects. Periods consisted of a 7-d (d 1 to 7) adaptation period followed by a 7-d (d 8 to 14) measurement period. On d 12, cows received an i.v. infusion of EPI (1.4 $\mu\text{g/kg}$ of BW). Blood was sampled at -45 , -30 , -20 , -10 , and -5 min before EPI infusion and 2.5, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min after. The AUC was calculated by incremental area, positive incremental area, and total area using the trapezoidal rule. The 3 methods resulted in different statistical inferences. When comparing the 3 methods for NEFA and glucose response, no significant differences among treatments and no interactions between treatment and AUC method were observed. For glucose and NEFA response, the method was statistically significant. Our results suggest that the positive incremental method and the total area method gave similar results and interpretation but differed from the incremental area method. Furthermore, the 3 methods evaluated can lead to different results and statistical inferences for glucose and NEFA AUC after an EPI challenge.

Key words: area under the curve, nonesterified fatty acid, glucose

Technical Note

The vitamin niacin has been described as a potential modulator of lipolytic responses in adipose tissue when administered in pharmacological doses (Carlson, 2005). However, niacin is known to be highly degradable in the rumen so that oral administration leads to

unknown quantities absorbed (Campbell et al., 1994). An experiment was conducted to determine responses of glucose and NEFA to epinephrine (EPI) challenge as affected by 3 levels of dietary protected niacin (PN) or unprotected niacin (UN) in the diet or infused abomasally. The response was measured by calculating the area under the curve (AUC) of sequential metabolite concentrations in plasma. Glucose and NEFA AUC displayed a quadratic response among treatments ($P = 0.03$) and ($P = 0.01$), respectively. Dietary PN unexpectedly resulted in greater lipid mobilization in response to EPI challenge compared with cows receiving equivalent dietary UN (Cardoso et al., 2011).

Many metabolic studies use AUC as a tool to assess the concentration of a metabolite over time, often after an experimental treatment or challenge. Different methods are used to calculate the AUC. The trapezoidal rule has been used as the standard method to measure AUC by summing the area of all the trapezoids formed between 2 time points (Chiou, 1978). Purves (1992) compared 11 different methods for calculating the AUC using synthetic data and found differences in the variance of their estimates. Allison et al. (1995) and Potteiger et al. (2002) demonstrated different results for glucose and insulin responses in human patients after an oral glucose tolerance test, depending on which AUC calculation method was chosen. Three methods are most often used: incremental area, where the positive and negative areas above or below the baseline are added; positive incremental area, where only positive areas above the baseline are included; and total area, which is obtained without the presence of a baseline, calculating the area below the observed concentrations.

In this study, we aimed to determine if these 3 methods for calculating AUC for glucose and NEFA response differ and whether any differences would affect interpretation of the outcomes of the treatments after an EPI challenge in dairy cows.

All experimental procedures were conducted according to protocols approved by the University of Illinois Institutional Animal Care and Use Committee. Six multiparous rumen-cannulated Holstein cows (BW =

Received June 16, 2011.

Accepted September 2, 2011.

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656 kg) after peak lactation (128 ± 23 DIM) were assigned to 1 of 6 treatments in a completely randomized 6×6 Latin Square design, with an extra period to measure carryover effects. Periods consisted of a 7-d (d 1 to 7) adaptation period followed by a 7-d (d 8 to 14) measurement period. Cows were milked 3 times daily, were housed in tie-stalls, and were fed individually throughout the experiment. Cows were fed according to NRC (2001) recommendations to meet or exceed recommendations. Treatments were no niacin (control, **CON**); abomasal infusion of 12 g/d of UN (**INF**); 12 g/d of UN in diet (**N12**); 3 g/d of PN in the diet (**BN3**); 6 g/d of PN in the diet (**BN6**); and 12 g/d of PN in the diet (**BN12**). For treatments N12, BN3, BN6, and BN12, niacin was top-dressed on the TMR twice daily. The daily dose of treatment INF was divided into 5 equal portions and infused every 4 h during the day. Cows receiving treatments other than INF were abomasally infused with the same volume of water at the same times. On d 11, a catheter (16-gauge, Abbocath-TAL catheter; Abbott Laboratories, North Chicago, IL) was placed in the jugular vein of each animal. On d 12, cows received an i.v. infusion of EPI ($1.4 \mu\text{g/kg}$ of BW). Blood was sampled at -45 , -30 , -20 , -10 , and -5 min before EPI infusion and 2.5, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min after. Analysis for NEFA was performed using the Wako NEFA-HR(2) Microtiter Procedure kit (Wako Diagnostics, Richmond, VA). Glucose was measured at the University of Illinois Veterinary Medicine pathobiology laboratory using automated methods according to Stein (1965).

Statistical analysis was performed using the MIXED procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC) with the following models:

$$Y_{ijk} = \mu + T_i + C_j + P_k + e_{ijk}, \quad [1]$$

where Y_{ijk} = an observation from the i th treatment, j th cow, in the k th period; μ = the grand mean; T_i = the fixed effect of the i th treatment; C_j = the random effect of the j th cow; P_k = the random effect of the k th period; and e_{ijk} = the random effect of the experimental error from the i th treatment, j th cow, in the k th period.

$$Y_{ijkl} = \mu + T_i + M_j + C_k + P_l + (T \times M)_{ij} + (T \times M \times C)_{ijk} + e_{ijkl}, \quad [2]$$

where Y_{ijkl} = an observation from the i th treatment, j th method to calculate AUC, for the k th cow, in the l th period; μ = the grand mean; T_i the fixed effect of the i th treatment; M_j = the fixed effect of the j th method to calculate the AUC; C_k = the random effect of the k th

cow; P_l = the random effect of the l th period; TM_{ij} = effect of the treatment by method interaction; TMC_{ijk} = random effect of the treatment by method by cow interaction; and e_{ijkl} = random effect of the experimental error from the i th treatment, j th method, k th cow, in the l th period. The TMC_{ijk} term is consistent with this model being a split-plot design, with method being split within the cow $\times$ treatment $\times$ period effect. Carryover effects were nonsignificant ($P > 0.45$) and were removed from the model. The number of lactations, BW, and DIM also were not significant ($P > 0.1$) in either model and were removed. Time points before the EPI injections ($n = 5$) were used to calculate a baseline for each animal. The 3 AUC calculation methods were performed using the trapezoidal rule (Purves, 1992). The effect of dietary treatments was calculated within each AUC method using Model 1. Model 2 was used to compare all 3 methods.

Least squares means were separated using the Tukey adjustment statement in SAS. The NEFA and glucose AUC responses in Model 2 were transformed to the natural log; this transformation showed to be the best option for normalization and homoscedasticity of the residuals. One outlier was identified as extreme (5 SD beyond the mean of the data set) and was removed. The Appendix provides SAS codes for AUC calculation methods.

The response of NEFA to EPI challenge is shown in Table 1. When AUC was calculated by the incremental area method, a trend ($P = 0.09$) for differences among treatments was detected. However, inferences would be different using the positive incremental area and total area methods. For AUC calculated by the incremental area method, a significant ($P < 0.05$) quadratic effect among BN3, BN6, and BN12 for NEFA response occurred. This effect was not detected when using the other 2 methods. For glucose AUC (Table 2), the statistical inference about differences among treatments would not change, regardless of calculation method. Nevertheless, the AUC calculated by positive incremental area suggested variation among treatments, which may be due to the presence of more negative values for the NEFA response relative to baseline than for the glucose response. Negative NEFA area values were most present at the end of the EPI stimulation period (60, 90, and 120 min; Figure 1).

It is important for investigators to determine how metabolites are affected by EPI challenge. The EPI injection clearly produced a peak of NEFA concentration around 15 min and then started to decrease after that (Figure 1). One can postulate that the area C in Figure 1 is simply an artifact of an acute NEFA response to EPI, which also led to high concentrations of glucose

Table 1. Means for NEFA response area under the curve (AUC; mEq/L per 120 min) after epinephrine injection among different niacin treatments¹

Calculation method	Overall <i>P</i> -value (treatment)	Treatment				
		CON	N12	INF	BN3	BN6
Incremental area	0.090	1.5958 ± 1.2655	3.1601 ± 1.3462	2.1418 ± 1.3426	1.2602 ± 1.3450	4.6845 ± 1.2655
Positive incremental area	0.412	3.4123 ± 1.1472	4.1010 ± 1.2171	4.9751 ± 1.2171	3.7407 ± 1.2188	5.3833 ± 1.1472
Total area	0.529	23.7510 ± 3.2044	21.7729 ± 3.3616	27.6627 ± 3.3674	22.4512 ± 3.3674	23.9310 ± 3.2044

¹CON = no niacin; INF = abomasal infusion of 12 g/d of unprotected niacin (UN); N12 = 12 g/d of protected niacin (PN); BN3 = 3 g/d of protected niacin (PN); BN6 = 6 g/d of PN; and BN12 = 12 g/d of PN (LSM ± SE).

Table 2. Glucose response area under the curve (AUC; mg/dL per 120 min) after epinephrine injection among different niacin treatments¹

Calculation method	Overall <i>P</i> -value (treatment)	Treatment				
		CON	N12	INF	BN3	BN6
Incremental area	0.283	2,117.75 ± 296.47	1,746.30 ± 320.01	2,451.89 ± 320.67	2,523.64 ± 320.67	1,885.04 ± 296.47
Positive incremental area	0.104	2,117.75 ± 236.22	1,788.00 ± 252.57	2,437.30 ± 253.10	2,509.05 ± 253.10	1,885.04 ± 236.22
Total area	0.720	9,291.67 ± 395.20	9,015.19 ± 428.64	9,683.05 ± 429.46	9,660.80 ± 429.46	9,604.79 ± 395.20

¹CON = no niacin; INF = abomasal infusion of 12 g/d of unprotected niacin (UN); N12 = 12 g/d of protected niacin (PN); BN3 = 3 g/d of protected niacin (PN); BN6 = 6 g/d of PN; and BN12 = 12 g/d of PN (LSM ± SE).

Figure 1. Graphical representation of 3 area under the curve (AUC) calculation methods for concentrations of NEFA after an epinephrine (EPI) challenge in lactating dairy cows. Incremental area = A + C; positive incremental area = A; total area = A + B. Baseline (0.262 mEq/L) = mean of 5 measurements before EPI injection at time 0. Values of NEFA are for an EPI challenge in 1 cow under treatment of 3 g/d of protected niacin in the diet (BN3) in period 3.

and is not a measure of lipolysis itself. In this case, the positive incremental method would lead to the more appropriate biological meaning when compared with the other 2 methods.

As an example, adding the baseline as a covariate when analyzing the total area data in model 1 was highly significant for both response variables ($P < 0.01$). The use of a covariate could control cow variation for this AUC calculation method that does not consider prior measurements as a baseline. The quadratic effect among BN3, BN6, and BN12 for NEFA AUC response was statistically significant for values calculated by the incremental area method.

Comparison of the 3 AUC methods for NEFA response showed no statistical difference among treatments and no interaction between treatment and method ($P > 0.1$). Method was statistically significant in the model ($P < 0.001$), which might be expected because the different methods result in a different scale of numbers. The total area calculation leads to larger values than either incremental or positive incremental area. The 3 methods of AUC calculation for NEFA response differed ($P < 0.05$).

Glucose AUC responses differed among methods ($P < 0.01$) but the effect of dietary treatment did not reach statistical significance ($P = 0.12$). The interaction of method by treatment also was not significant ($P > 0.1$). The incremental area and positive incremental area methods were similar to each other ($P > 0.1$). However, both methods were different from the

total area calculation ($P < 0.01$). The baseline used in the calculation of AUC by these methods affected the interpretation when used as a covariate, probably controlling for between-cow variation.

The method used to calculate the AUC responses of NEFA and glucose to an EPI challenge in dairy cows may lead to different inferences about the effects of experimental treatments. The suitability of a specific method may vary among different animal systems and response variables. Investigators should report the specific method of calculation when reporting values for AUC responses to lead to a better understanding of sources of variation. Further investigation of the total area method with use of the baseline as covariate should be considered, perhaps by using synthetic data sets with known expected biological responses. Our results indicate that the positive incremental area and the total area methods provided similar results and statistical interpretations as the gold standard incremental area method. However, the 3 methods evaluated can lead to different results and different statistical inferences for the response variables glucose and NEFA AUC after an EPI challenge.

ACKNOWLEDGMENTS

The authors are grateful to QualiTech Inc. (Chaska, MN) for the supply of the protected and unprotected niacin sources and for partial financial support.

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APPENDIX

The SAS statements to produce the calculation of the AUC for 3 approach methods are:

(1) Incremental area:

```
Data Both; SET Both; TP1 = LAG(TP); N1 =
LAG(NEFA); AVE = (NEFA +N1)/2-BASE; LNG = TP-
TP1;
AREA = AVE*LNG;
Data BOTH; SET BOTH; IF TP LT 3 THEN DELETE;
PROC SUMMARY SUM; VAR AREA; BY COW PERIOD
TRT; OUTPUT OUT = AREA SUM = AUC;
RUN;
```

(2) Positive incremental:

```
Data Both; SET Both; TP1 = LAG(TP); N1 =
LAG(NEFA); AVE = (NEFA+N1)/2-BASE; LNG = TP-
TP1;
AREA = AVE*LNG;
Data Both; SET Both; IF TP LT 3 THEN DELETE;
IF AREA < 0 THEN AREA = .;
RUN;
PROC SUMMARY SUM; VAR AREA; BY COW PERIOD
TRT; OUTPUT OUT = AREA SUM = AUC;
RUN;
```

(3) Total area:

```
Data Both; SET Both; TP1 = LAG(TP); N1 =
LAG(NEFA); AVE = (NEFA+N1)/2; LNG = TP-TP1;
AREA = AVE*LNG;
Data Both; SET Both; IF TP LT 3 THEN DELETE;
IF AVE < 0 THEN AVE = .; IF AREA < 0 THEN
AREA = .;
RUN;
PROC SUMMARY SUM; VAR AREA; BY COW PERIOD
TRT; OUTPUT OUT = AREA SUM = AUC;
RUN;
```

TP: time point where the metabolite concentration (NEFA/glucose) was measured; TP1: difference of time (minutes) between 2 time points; NEFA: metabolite concentration; N1: difference of metabolite concentration between 2 time points; BASE: mean of 5 time points before EPI injection (baseline); AREA: area of one trapezoid; AUC: area under the curve.