Effect of sub-clinical uterine infection on plasma amino acid concentrations in early lactation dairy cows

T. LOPDELL[1], M.C. BERG[2], M.P. GREEN[2] and P.J. BACK[1]*

[1]Livestock Improvement Corporation, Private Bag 3016, Hamilton 3240, New Zealand
[2]AgResearch Ruakura, Private Bag 3123, Hamilton 3240, New Zealand
*Corresponding author: p.j.back@massey.ac.nz

ABSTRACT

This study examined the effect of sub-clinical endometritis (scEndo) in early lactation on plasma amino acid (AA) concentrations of dairy cattle (n = 46). On D21 and D42 postpartum cows were classified as having scEndo or Clean based on uterine cytology; >18% polymorphonuclear (PMN) cells amongst uterine nucleated cells. On D21 and D42, 35% and 7% of cows respectively were classified as having scEndo, with a large proportion of scEndo cows having self-resolved the infection by D42. Plasma serine concentrations were higher ($P < 0.03$) in scEndo compared to Clean cows irrespective of day postpartum. Independent of uterine status, three AA altered ($P < 0.05$) in concentration between D21 and D42. The effect of change in uterine status from D21 to D42 resulted in decreased ($P = 0.05$) concentrations of three AA in cows that resolved infection. The use of a lower cut-off to define scEndo (4% vs 18%PMN) identified four AA, instead of three AA at 18% that differed over time. Thus, during the early postpartum period, the concentrations of a number of AA change ($P < 0.05$) irrespective of uterine health. However, scEndo is associated with an increase in plasma serine and a decrease in the concentration of several AA when a uterine infection is resolved.

Keywords: amino acid; plasma; sub-clinical infection; uterine; dairy cow.

INTRODUCTION

The seasonal nature of the New Zealand dairy system requires cows to conceive and produce a calf every 365 days. However, sub-fertility caused by a number of factors including inappropriate management, nutrition and disease often results in calving intervals of >365 days (Gordon, 1996). A disease that commonly affects dairy cows is endometritis (Le Blanc, 2008), caused by a bacterial infection that leads to inflammation of the uterine endometrium (Kasimanickam *et al.*, 2004). Endometritis is associated with several reproductive problems, such as lower conception rates and longer intervals between calving and conception (Williams *et al.*, 2005) and is usually estimated to affect approximately 15% of cows (Williams *et al*., 2005; Potter *et al*., 2010). In addition to clinical endometritis, sub-clinical endometritis (scEndo), is a substantial issue, with a prevalence as high as 30-50% in some herds (Le Blanc, 2008). However, scEndo often remains undetected, due to the lack of obvious symptoms, so its impact on milk yield and reproduction are poorly understood (Le Blanc, 2008).

Disease and infection can lead to the repartitioning of vital nutrients such as amino acids (AA) within the body to fight these detrimental conditions (Askanazi *et al.*, 1980; Vente *et al*., 1989). Unfortunately, this is often at the expense of processes deemed as additional to basic maintenance requirements such as reproduction. Hence the measurement of circulating concentrations of AA and other nutrients may be useful in potentially assessing available resources required for reproduction.

Amino acids can affect fertility at different stages of the reproductive process, namely within the ovary, affecting oocyte quality (Rooke *et al*., 2009), as well as within the uterine environment, influencing embryo growth and viability (Sinclair *et al.*, 2008). AA are also converted into metabolites which are important for fertility. Arginine, for example, can be converted into nitric oxide, which is an important signalling molecule in many pathways (Wu, 2009). As well as influencing fertility, AA are required by the immune system. Some, such as asparagine, arginine, glutamine and threonine, are involved directly, while other AA are used to produce molecules with immune functions, such as glutathione from glutamine and anthranilic acid from tryptophan (Wu, 2009).

The objective of this study was to examine the effects of scEndo on the concentrations of amino acids in the plasma of early postpartum dairy cattle, based on the hypothesis that scEndo has a substantial negative impact on the partitioning of nutrients crucial for the reproduction of cows in pasture-grazed farming systems. Ultimately, the goal of this research is the early diagnosis and treatment of scEndo to alleviate potential negative impacts upon reproduction.

MATERIAL AND METHODS

This study was approved by the Ruakura Animal Ethics Committee and conducted from

August to October 2008. Cow management was as described by Green *et al.* (2009). Cows included in the trial were multiparous, exhibited general good health and had not been treated with intra-mammary or systemic antibiotics post-calving. Cows that exhibited signs of clinical uterine or mammary infection were treated and excluded from the trial.

The uterine health status of mixed-age lactating dairy cows (n = 46) was assessed by determination of percentage of polymorphonuclear neutrophils (%PMN) in the uterus. Uterine endometrial samples were taken on D21 and D42 postpartum (standard error ± 3D) for cytological analysis using a cytobrush technique (Kasimanickam *et al.*, 2005; Barlund *et al.*, 2008) and the %PMN determined at the Animal Health Centre, Morrinsville. On D21 and D42, cows with >18 %PMN were classified as having scEndo (Kasimanickam *et al.*, 2005).

Blood samples were taken by venipuncture of the coccygeal vein on D21 and D42 postpartum (heparinised vacutainers, Becton Dickenson, Auckland). Samples were centrifuged for 15 minutes at 1,500 *g* at 4°C and the resulting plasma stored at -20°C until analysis. Concentrations of 21 amino acids, namely alanine, arginine, asparagine, aspartate, citrulline, glutamate, glutamine, glycine, histidine, hydroxy-proline, isoleucine, leucine, lysine, ornithine, phenylalanine, proline, serine, taurine, threonine, tyrosine and valine, were determined using a florescence-tag high performance liquid chromatography (HPLC) method (Bloomfield *et al.,* 2002).

TABLE 1: Mean ± standard error of plasma amino acid concentrations (µmol/L) of dairy cows irrespective of uterine status 21 days (D21) and 42 days (D42) postpartum.

Amino acid	D21	D42	P value
Number of cows	46	45	
Alanine	210.3 ± 9.3	208.9 ± 9.8	0.90
Arginine	55.3 ± 3.1	52.0 ± 3.3	0.42
Asparagine	39.6 ± 1.5	38.9 ± 1.6	0.73
Aspartate	7.6 ± 1.3	10.7 ± 1.4	0.08
Citrulline	97.8 ± 3.2	99.3 ± 3.4	0.68
Glutamate	34.6 ± 2.5	43.1 ± 2.7	**0.03**
Glutamine	226.3 ± 8.4	190.5 ± 8.9	**<0.01**
Glycine	625.8 ± 26.0	599.4 ± 27.4	0.39
Histidine	33.0 ± 1.7	31.7 ± 1.8	0.57
Isoleucine	108.0 ± 3.5	100.2 ± 3.7	0.07
Leucine	123.9 ± 4.3	112.2 ± 4.5	**0.04**
Lysine	84.4 ± 4.5	75.8 ± 4.8	0.14
Hydroxyproline	8.9 ± 0.3	7.5 ± 0.4	**<0.01**
Ornithine	25.4 ± 3.4	32.6 ± 3.6	0.16
Phenylalanine	51.7 ±1.7	49.7 ± 1.8	0.36
Proline	88.4 ± 3.2	82.4 ± 3.3	0.08
Serine	113.9 ± 9.1	139.5 ± 9.7	0.06
Taurine	27.4 ± 1.7	29.0 ± 1.8	0.08
Threonine	105.6 ± 4.7	106.5 ± 5.0	0.88
Tyrosine	52.4 ± 2.3	53.1 ± 2.4	0.81
Valine	224.4 ± 7.7	217.5 ± 8.0	0.37

Statistical analyses

Plasma AA data were analysed using two methods. Firstly, to test for an effect of uterine status or day postpartum on plasma AA concentration, the PROC MIXED function in SAS version 9.1 1 (SAS Institute Inc., Cary, NC, USA) was employed. Cows were assigned their uterine status (Clean or scEndo) based on their D21 %PMN examination. The model included uterine status and day postpartum as fixed effects with cow as a random effect. Secondly, changes in plasma AA concentrations of cows in which sEndo self-resolved where the uterine status on D21 from scEndo changed to clean on D42 postpartum, were investigated. Cows were classified as Clean (clean on both days), scEndo (scEndo on both days) or Resolved (scEndo on D21 but clean on D42). These data were analysed using the Kruskal-Wallis (KW) rank sum test in R version 2.11 (R Core Development Team, 2010). In addition, since debate exists in the literature on the most relevant %PMN threshold to use to define scEndo, data were analysed and compared using both 18% (Kasimanickam, 2004) and 4% PMN, the median of the %PMN for D21 and D42 combined in the current study).

RESULTS

Prevalence of scEndo

Uterine cytological examinations of 46 (D21) and 45 (D42) cows resulted in 16 of 46 (35% at D21) and three of 45 (7% at D42) cows classified as having scEndo using an 18%PMN threshold. One cow developed clinical endometritis and was removed from the study by D42. The proportion of scEndo cows that had self-resolved the infection by D42 was 13 of 16 (81%) with an 18% PMN threshold, whilst 58% at D21 and 23% at D42 of cows were classified as having scEndo using a 4% PMN threshold. The proportion of these cows that had self-resolved the infection by D42 was 15 of 26 (58%).

Changes in AA concentration

No effect of D21 uterine status was found on plasma AA concentrations with the exception of serine (Clean, 110.6 ± 8.3 vs scEndo, 139.5 ± 9.7 µmol/L; P <0.03) and aspartate (Clean, 7.3 ± 1.2 vs scEndo, 10.7 ± 1.4 µmol/L; P = 0.08). Day postpartum affected (P <0.1) a number of AA concentrations (Table 1). Between D21 and D42 three AA concentrations decreased (P <0.05) and five tended (P <0.1) to change in concentration.

TABLE 2: Mean ± standard error of plasma AA concentrations (μmol/L) for Clean (n = 27) and scEndo (n = 13) cows 21 days (D21) postpartum, plus changes in concentration between D21 and 42 days (D42) postpartum for cows reclassified as Clean (<18%PMN) (n = 27), scEndo (>18%PMN) (n = 3) or Resolved (<18%PMN) (n = 10) 42 days (D42) postpartum. P values show the KW test for equality of medians between the three D42 groups. Bolding of P values indicates significant differences (P <0.05) between the three D42 groups.

Amino acid	D21		D42			P value
	Clean	scEndo	Clean	scEndo	Resolved	
Alanine	228.2 ± 9.2	231.8 ± 12.5	9.5 ± 11.6^{a}	62.7 ± 23.1^{b}	-13.0 ± 8.8^{b}	0.08
Asparagine	20.8 ± 1.7	21.9 ± 1.6	3.2 ± 1.5^{a}	12.7 ± 0.7^{b}	1.6 ± 2.1^{ac}	**0.02**
Phenylalanine	50.1 ± 2.0	52.0 ± 2.5	5.0 ± 2.0^{a}	10.8 ± 4.9^{ab}	-6.8 ± 6.6^{ac}	0.08
Serine	124.9 ± 6.0	129.5 ± 9.0	22.1 ± 5.6^{a}	56.8 ± 9.7^{ab}	12.0 ± 7.5^{ac}	0.06
Threonine	96.1 ± 6.7	99.7 ± 7.7	8.0 ± 5.6^{a}	42.0 ± 5.9^{ab}	-14.0 ± 12.4^{ac}	**0.02**
Tyrosine	47.9 ± 2.5	47.9 ± 4.5	6.6 ± 3.6^{a}	26.1 ± 5.4^{b}	0.7 ± 3.9^{ac}	0.06
Valine	241.1 ± 10.7	237.1 ± 15.8	-1.3 ± 7.6^{a}	42.2 ± 8.3^{b}	-14.8 ± 11.7^{ac}	**0.05**

TABLE 3: The mean ± standard error of the mean for change in plasma amino acid concentrations (μmol/L) between 21 days (D21) and 42 days (D42) postpartum in cows classified as having sub-clinical endometritis (scEndo) using two different thresholds; 4 or 18% polymorphonuclear (PMN) cells amongst uterine cells. Cows were classified as "Clean" if their %PMN was less than the classification threshold on both D21 and D42, "scEndo" if their %PMN was greater than the classification threshold on both D21 and D42, and "Resolved" if their %PMN was greater than the classification threshold on D21 but less than the threshold on D42. n = Number of cows in group. Bolding of P values indicates significance (P <0.05) in amino acid concentrations within a %PMN classification. Subscript letters identify significant (P <0.05) differences between groups within a %PMN classification.

Amino acid	4% PMN				18% PMN			
	Clean (n = 14)	scEndo (n = 6)	Resolved (n = 17)	P-value	Clean (n = 27)	scEndo (n = 3)	Resolved (n = 10)	P value
Alanine	20.4 ± 16.5^{a}	30.6 ± 18.6^{ab}	-18.3 ± 10.1^{ac}	**0.05**	9.5 ± 11.6^{a}	62.7 ± 23.1^{ab}	-13.0 ± 8.8^{ac}	0.08
Asparagine	1.4 ± 2.3^{a}	8.4 ± 2.8^{bc}	2.8 ± 1.6^{ac}	0.11	3.2 ± 1.5^{a}	12.7 ± 0.7^{ab}	1.6 ± 2.1^{ac}	**0.02**
Isoleucine	-5.4 ± 6.3^{a}	23.2 ± 4.2^{b}	-9.0 ± 5.6^{ac}	**0.01**	-2.4 ± 4.7^{a}	23.6 ± 3.2^{ab}	-7.6 ± 7.0^{ac}	0.29
Leucine	-4.6 ± 7.0^{a}	27.7 ± 10.2^{b}	-10.8 ± 8.7^{ac}	**0.03**	-2.4 ± 6.0^{a}	18.4 ± 6.4^{a}	-7.5 ± 13.1^{a}	0.36
Phenylalanine	4.3 ± 2.9^{a}	10.8 ± 3.4^{ab}	-3.0 ± 4.2^{ac}	0.09	4.9 ± 2.0^{a}	10.8 ± 4.9^{ab}	-6.8 ± 6.6^{ac}	0.08
Serine	17.7 ± 8.4^{a}	33.1 ± 16.4^{a}	23.1 ± 5.6^{a}	0.43	22.1 ± 5.6^{a}	56.8 ± 9.7^{ab}	12.0 ± 7.5^{ac}	0.06
Threonine	5.2 ± 9.1^{a}	27.0 ± 8.3^{ab}	-4.7 ± 8.5^{ac}	0.09	8.0 ± 5.6^{a}	42.0 ± 5.9^{b}	-14.0 ± 12.4^{ac}	**0.02**
Tyrosine	5.1 ± 5.3^{a}	19.0 ± 5.5^{ab}	1.8 ± 3.1^{ac}	0.07	6.6 ± 3.6^{a}	26.1 ± 5.4^{ab}	0.6 ± 3.9^{ac}	0.06
Valine	-1.2 ± 11.9^{a}	35.9 ± 9.0^{ab}	-14.4 ± 7.1^{ac}	**0.01**	-1.3 ± 7.6^{a}	42.2 ± 8.3^{b}	-14.8 ± 11.7^{ac}	**0.05**

In cows that self-resolved between D21 and D42, three AA concentrations differed significantly at the 5 % level, and four tended at the 10% level, to differ amongst the D42 Clean, scEndo and Resolved cows (Table 2). These AA showed similar patterns of change; the concentrations in the Clean and Resolved cows increased slightly, while those of scEndo cows increased dramatically. No AA showed differences between the concentration changes in Clean and Resolved cows.

When the %PMN threshold for defining scEndo on both D21 and D42 was lowered from 18% to 4%, seven of the AA studied were significant at the 10% level; three of these, namely the three branched chain AA of leucine, isoleucine and valine, were significant at the 5% level (Table 3). Neither leucine nor isoleucine was significant (P <0.1) using an 18% threshold.

DISCUSSION

The current study identified, firstly that plasma amino acid concentrations changed over time (between D21 and D42 postpartum), secondly that sub-clinical endometritis was associated with altered circulating concentrations of selected AA during early lactation in pasture-fed dairy cows, thirdly that AA concentrations of cows in which an infection self-resolved by D42 postpartum were found to return to those of 'clean' cows, and fourthly that the PMN% threshold chosen to distinguish between infected and uninfected cows influences how

differences in amino acid concentrations might be used to further study uterine health in the early-lactation dairy cow.

This study determined that large variations exist in circulating AA concentrations of lactating dairy cows, between both AA and cows; a finding that is consistent with previous published literature, in both overseas and New Zealand studies (Meijer *et al.,* 1995; Blum *et al.,* 1999; Pacheco-Rios *et al.,* 1999; Back, 2002). In these studies, AA were predominantly influenced by diet and stage of lactation. For example, concentrate-fed animals had AA concentrations that were approximately 20% lower (Blum *et al*., 1999) or 15% higher (Meier *et al*., 1995) than reported here. Pasture-fed cows in early lactation had more similar concentrations. Those in Back (2002) were 12% lower, while those in Pacheco-Rios *et al.* (1999) were within 1% of this study.

Uterine status, specifically sub-clinical endometritis, was associated with an increased concentration of plasma serine and aspartate compared to clean animals. Both these amino acids have known immune and energetic functions. Serine is involved in the regulation of IL-2 production and T-lymphocyte activation (Li *et al.,* 2007) and gluconeogenesis (Wu, 2009); whereas aspartate has roles in leucocyte and lymphocyte function and metabolism, and is a precursor for other AA namely, methionine, threonine, isoleucine and lysine, which are required for gluconeogenesis (Li *et al.,* 2007). The latter role is corroborated by an elevated threonine concentration on D42 in scEndo compared to Clean cows.

Independent to changes associated with uterine status, an effect of day postpartum was evident for glutamate, glutamine, hydroxyproline and leucine. Again, immune functions are a reflection of a cow's general health and ability to fight infection. Recovery from the stresses of calving and adaptation to lactation may be explained by changes in the concentration of these AA, as glutamate, glutamine and leucine all have reported immune functions (Wu, 2009). In the present study concentrations of glutamine and leucine decreased between D21 and D42, while glutamate increased.

The mixed-model analysis, with day-postpartum as the treatment effect, assumed all cows had the same scEndo status throughout the experiment. However, a large proportion of the cows resolved their sub-clinical infection spontaneously by D42. Therefore, a second method, based on the KW test, was devised to take the resolutions into account. The KW method is non-parametric, so it is more robust when used with small sample sizes, or when outliers are present. Using day postpartum as the treatment effect in a mixed model gave a similar result to the KW test. The mixed model test showed valine, alanine, asparagine, aspartate, serine, hydroxyproline and ornithine concentrations to differ significantly between D21 and D42, along with tyrosine and glutamine at the 10% level. This compares to the KW test, which had alanine, serine, tyrosine and valine at the 10% level over the period D21 to D42. This illustrates the problem of using only the initial D21, uterine status when the majority of cows self-resolved during the trial. The KW method avoided this problem by classifying the cows into three groups, treating the Resolved cows separately.

Because of the high self-resolve rates in the scEndo cows from D21 to D42, only a small number, being nine at a 4% threshold and three at 18%, of cows had scEndo by D42. This problem is compounded by the large variation between cows in their plasma AA concentrations. Therefore, it is difficult to correctly determine which AA have significant differences in concentration changes between the "clean" and "resolved" groups of cows and a larger study is likely to be needed to find any differences, should they exist.

This study also investigated the implications of utilising two thresholds to define scEndo, both previously used by other studies. Debate over the %PMN threshold exists due to the divergence in the timing of uterine sampling, since endometrial inflammation, especially in the first three to four weeks postpartum, is part of normal uterine involution. The present study employed the threshold used by Kasimanickam *et al.* (2004). These authors defined the threshold for scEndo to be >18% for D20–33 postpartum and >10% for D34–47, which were determined using a response operating characteristics (ROC) curve for median days between calving and pregnancy. In the only other pasture-based study, Burke *et al*. (2010) used a threshold being the lowest value of the upper quartile, of >6% at D42 for defining infected cows and this was associated with reduced fertility. In overseas studies, Barlund *et al.* (2008), using the lowest percentage to be significantly associated with pregnancy status at 150 days, determined a threshold of >8% at D28 to D41. Other studies have used a range of thresholds, depending on the number of days postpartum at which samples were collected: >25% at D28 (Hammon *et al.*, 2006), >5% between D40-60 (Gilbert *et al.*, 2005), and ≥3% at D190 (Salasel *et al.*, 2010).

Because of this disagreement, two different thresholds were used in this study: 18%PMN and 4%PMN. The number of AA identified to change between Clean and scEndo cows were the same, however, agreement between the specific AA was only at approximately 70%. This emphasises the difficulty in using arbitrary thresholds unrelated to subsequent reproductive outcomes, although this result may partly be explained by the low number of cows within the study, especially scEndo cows on

D42 postpartum. Despite this, the 70% agreement using the two divergent thresholds does indicate there to be merit in focussing on the AA identified as being common in both analyses. Large scale retrospective studies in which cows are frequently sampled throughout the postpartum period are therefore advocated by the authors of the current study.

In conclusion, scEndo is associated with plasma AA concentrations of dairy cows in early lactation. While scEndo appeared to have little effect on circulating AA concentrations at D21 as determined by the mixed-model, the ability of the cow to resolve scEndo does appear to be associated with a change in circulating concentrations of AA by D42, with concentrations often dropping to similar levels to those in clean cows once the scEndo resolves (Table 2). This is in addition to the concurrent increase in concentration of many AA in scEndo cows between D21 and D42 postpartum. Further studies may thus be warranted to investigate the potential usefulness of monitoring AA changes in early postpartum as a useful proxy for the availability of AA to increase the chance of successful reproduction.

ACKNOWLEDGEMENTS

The authors would like to thank AgResearch farm staff for assistance with data collection, as well as Eric Thorstensen, University of Auckland, for undertaking amino analysis. Partial funding was received from DairyNZ (AN808) and FRST (Contract UOAX0814).

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