Sub-clinical uterine infection is associated with altered amino acid concentrations of follicular fluid in early lactation dairy cows

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ABSTRACT

The effect of sub-clinical endometritis (scEndo) in early lactation on follicular amino acid (AA) concentrations was determined. 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. Follicular histidine ($P = 0.04$), as well as alanine, aspartate and serine concentrations ($P < 0.1$) were higher in scEndo compared with Clean cows. The effect of change in uterine status from D21 to D42 resulted in increased ($P < 0.1$) follicular glutamate, ornithine and valine concentrations in cows that resolved infection. Correlation coefficients were determined to test the relationship between plasma and follicular AA concentrations. A greater number of AA in Resolved cows showed no relationship between plasma and follicular concentrations compared to Clean cows. Thus, scEndo is associated with an increase in follicular histidine and an increase in the concentration of several AA when a uterine infection is resolved. This study demonstrates that scEndo may be responsible for long-term alterations in follicular AA concentrations that could potentially result in altered oocyte viability.

Kewords: follicle; amino acid; sub-clinical infection; dairy cow.

INTRODUCTION

An early resumption of oestrous cycles and rapid uterine involution during the early postpartum period are required to maintain a 365-day calving interval in New Zealand dairy cows. The success of this process is negatively affected by uterine infection, which can manifest itself either sub-clinically or clinically and is associated with the reduced fertility of dairy cows. This is evident as longer intervals from calving to first insemination or conception for infected cows, with more cows culled for failure to conceive within a required time frame (McDougall, 2001; LeBlanc 2002; Kasimanickam *et al.*, 2004; Burke *et al.*, 2010). Few studies however have examined the effect of sub-clinical endometritis (scEndo) on fertility, although Burke *et al.* (2010) determined reduced fertility in infected cows compared to controls and Kasimanickam *et al.* (2004) identified scEndo cows had an increased interval between parturition and pregnancy and lower conception rates.

Nutrition and infection are several of the major factors that affects fertility by numerous mechanisms that can ultimately reduce the quality of the oocyte ovulated (Beisel, 1975; Rooke *et al*., 2009). Specifically, lactation, negative energy balance (a catabolic state) or disease can cause marked changes in circulating amino acids (AA) (Field *et al.,* 2002; Wu, 2009). This may be important in cows with a sub-clinical disease such as scEndo as the follicular fluid contained within the ovarian follicle derives its components from plasma and locally produced substances that are related to the metabolic activity of the follicular cells (Gosden *et al.,* 1988; Gérard *et al.,* 2002). Thus, the systemic nutrient concentrations affect the concentrations in the follicular fluid surrounding the oocyte in the ovary (Gosden *et al*., 1988), which in turn influence the competency of the oocyte (Orsi *et al.*, 2005).

Hence, AA concentrations in follicular fluid may be important for the competency of the developing oocyte. The importance of the AA profile of follicular fluid is highlighted by a comparison of pre-ovulatory follicular fluid and oocyte *in vitro* maturation (IVM) medium (Orsi *et al.,* 2005), where concentrations in media of all AA, except histidine, alanine, tryptophan and taurine, were consistently higher than in follicular fluid; potentially accounting for the poor developmental competence of IVM oocytes. Another study by Sinclair *et al*. (2008), suggested that AA composition of follicular fluid, particularly alanine and glycine, can be used as a predictor of embryo development.

Previously we have demonstrated that scEndo in pasture-fed cows during early lactation altered haematological (neutrophil parameters, red blood cell count and haemoglobin concentrations) and plasma biochemical (albumin), as well as milk (lower fat and protein concentrations) parameters (Green *et al.,* 2009). The objective of the present study was to ascertain whether the amino acid (AA) profile in follicular fluid was altered in pasture-fed dairy cows with a sub-clinical uterine infection as

compared to those with a healthy uterus. Ultimately, this research will help to ascertain if perturbation in nutrients, such as AA can explain the sub-fertility associated with scEndo in dairy cows.

MATERIALS 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 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 the percentage of polymorphonuclear neutrophils (%PMN) in the uterus. Uterine endometrial samples were taken on Day 21 (D21) and Day 42 (D42) postpartum (± 3D) for cytological analysis using a cytobrush technique (Kasimanickam *et al.*, 2005; Barlund *et al.*, 2008) and the %PMN determined (Animal Health Centre, Morrinsville). On D21, cows with >18%PMN were classified as having scEndo (Kasimanickam *et al.*, 2005).

Blood samples were taken by venipuncture of the coccygeal vein on D42 and D63 postpartum into heparinised vacutainers (Becton Dickinson, Auckland). Samples were centrifuged for 15 minutes at 1500 *g* at 4°C and the resulting plasma stored at –20°C until analysis.

Follicular and luteal dynamics were evaluated by ultrasonography as described by Back *et al.* (2009). Briefly, follicular populations were mapped using a 7.5 mHz transvaginal sector probe (PieMed 200S, Maastricht, The Netherlands) and the diameters of individual follicles measured using the internal callipers of the ultrasound system. On D42 and D63 postpartum, all follicles larger than 4 mm were ablated using 19Gx1.5"BD Precision-Glide needles (Becton Dickinson, Auckland, New Zealand) with 25 mm Hg vacuum using an aspiration pump (Karl Storz, Germany) and four days later the diameters of the new lead and sub-ordinate follicles were measured. Lead follicles were defined as the largest follicle on either ovary at assessment that eventually would have become the pre-ovulatory dominant follicles. From the lead follicle, follicular fluid was collected under vacuum, as described above, and stored on ice until centrifuged at 3000 *g* for 5 minutes at 4°C to remove follicular cells. The resulting supernatant was recovered and stored at –80°C for amino acid analysis. Follicular fluid concentrations of 21 amino acids were determined by a florescence-tag high Performance liquid chromatography (HPLC) method (Bloomfield *et al.,* 2002).

Statistical analyses

Follicular AA data were analysed using two methods. Firstly, to test for an effect of uterine status or day postpartum on follicular AA concentration, the PROC MIXED function in SAS version 9.1 was employed. Cows were assigned their uterine status (Clean or scEndo) based on their D21 %PMN results. The model included uterine status and day postpartum as fixed effects, cow as a random effect and follicle size as a covariate.

Secondly, changes in follicular AA concentrations of cows in which sEndo self-resolved (changing uterine status on D21 from scEndo 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).

TABLE 1: Mean ± standard error of the mean of the amino acid concentration in follicular fluid (μmol/L) of dairy cows pooled over Day 42 and Day 63 post-partum for cows with their uterine status classified as clean (<18%PMN) or scEndo (>18%PMN) based on D21 postpartum %PMN results. Bolding indicates significance (P <0.05). %PMN = Percentage of polymorphonuclear neutrophils in the uterus.

Amino acid	Clean	scEndo	P value
Number of cows	22	14	
Alanine	193.0 ± 8.7	221.2 ± 11.2	0.06
Arginine	48.5 ± 2.9	56.7 ± 3.8	0.10
Asparagine	35.7 ± 1.8	38.7 ± 2.3	0.31
Aspartate	7.1 ± 1.7	12.0 ± 2.2	0.10
Citrulline	93.6 ± 3.9	94.8 ± 4.1	0.82
Glutamate	39.3 ± 1.7	44.1 ± 2.2	0.11
Glutamine	175.6 ± 8.5	187.0 ± 10.9	0.42
Glycine	599.7 ± 27.1	564.4 ± 34.9	0.43
Histidine	27.8 ± 1.6	33.5 ± 2.1	**0.04**
Hydroxy-proline	6.7 ± 0.3	7.0 ± 0.4	0.53
Isoleucine	103.5 ± 3.8	101.4 ± 4.9	0.74
Leucine	112.2 ± 4.0	113.8 ± 5.2	0.80
Lysine	72.0 ± 4.2	81.9 ± 5.4	0.16
Ornithine	26.1 ± 4.2	37.9 ± 5.5	0.11
Phenylalanine	46.9 ± 1.3	50.3 ± 1.7	0.12
Proline	72.3 ± 3.3	81.8 ± 4.3	0.41
Serine	114.5 ± 11.9	148.5 ± 15.3	0.09
Taurine	24.5 ± 2.1	27.7 ± 2.7	0.36
Threonine	97.8 ± 4.8	110.3 ± 6.2	0.13
Tyrosine	47.6 ± 2.3	48.1 ± 2.4	0.20
Valine	218.9 ± 7.7	220.6 ± 9.9	0.90

Correlations were used to investigate relationships between plasma and follicular AA concentrations on D42 for both Clean and Resolved cows. Correlations were calculated using the Spearman method in R version 2.11 (R Core Development Team, 2010). Only samples from follicles free from blood contamination were used for these analyses.

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%).

Follicular characteristics

Uterine status (Clean vs scEndo) had no effect on the size or growth over four days of lead or sub-ordinate follicles (Back *et al.,* 2009). Follicular fluid samples were successfully collected without blood contamination on D42 and D63 from 31 of 46 (67%) and 36 of 45 (80%) cows respectively. On D42, data were analysed from 11 of 16 (69%) scEndo and 20 of 30 (67%) Clean cows, and on D63, 14 of 16 (88%) scEndo and 22 of 30 (73%) Clean cows.

Change in plasma amino acid concentrations

Results of this analysis are detailed in Lopdell *et al.* (2011). Briefly, plasma serine ($P < 0.03$) and aspartate ($P = 0.08$) concentrations were found to be higher in scEndo compared to Clean cows. Three AA, glutamine, hydroxy-proline and lysine, decreased ($P < 0.05$) between D21 and D42 postpartum. Concentrations of five other AA tended ($P < 0.1$) to alter in concentration. These were aspartate, isoleucine, proline, serine and taurine. In cows that scEndo self-resolved between D21 and D42 plasma asparagine, threonine, and valine concentrations decreased ($P < 0.05$) to concentrations similar to those of Clean cows.

Change in follicular amino acid concentrations

When D42 and D63 data were combined follicular histidine concentrations, were higher ($P = 0.04$) in scEndo compared to Clean cows, while alanine, aspartate and serine concentrations tended ($P < 0.1$) to increase in scEndo cows (Table 1). Day postpartum did not affect AA concentrations, aside from two AA that decreased between D42 and D63; hydroxy-proline (D42, 7.44 ± 0.31 μmol/L versus D63, 6.19 ± 0.29 μmol/L; $P = 0.002$) and citrulline (D42, 98.27 ± 3.17 μmol/L versus D63, 90.19 ± 2.96 μmol/L; $P = 0.02$).

The uterine status (scEndo or Clean) classification was based on %PMN determined on D21 and D42. Table 2 shows the combined mean of D42 and D63 follicular AA concentrations of Clean (clean on all days, n = 22), and scEndo resolved (scEndo on D21 but clean by D42, n = 14) cows. In cows that self-resolved between D21 and D42, follicular aspartate increased ($P < 0.01$), and three AA, namely glutamate, ornithine and valine, tended ($P < 0.1$) to increase (Table 2) compared with Clean

TABLE 2: Mean ± standard error of the mean of the amino acid concentration in follicular fluid (μmol/L) of dairy cows Day 42 post-partum and the change in concentration between Day 42 and Day 63 postpartum for cows reclassified as clean (<18%PMN) or scEndo resolved (<18%PMN) on Day 63 postpartum. P values show the KW test for equality of medians between the three groups. Superscripts within columns indicate a difference ($P < 0.05$) between changes in concentrations in clean and resolved cows. %PMN = Percentage of polymorphonuclear neutrophils in the uterus.

Amino acid	Day 42	Day 63		P value
	All cows	Reclassified as clean	Reclassified as resolved	
Number of cows	21	18	3	
Alanine	204.9 ± 50.5	-2.9 ± 44.7	-5.2 ± 54.3	0.69
Arginine	15.2 ± 13.8	0.5 ± 17.1	14.1 ± 14.6	0.16
Asparagine	38.3 ± 8.5	-3.3 ± 8.9	-5.8 ± 6.1	0.76
Aspartate	10.0 ± 11.4	-1.3 ± 2.3[a]	5.3 ± 4.4[b]	**<0.01**
Citrulline	97.2 ± 20.0	-7.5 ± 13.5	4.0 ± 2.4	0.11
Glutamate	41.2 ± 12.2	-3.9 ± 10.7[a]	7.6 ± 4.4[b]	0.07
Glutamine	188.7 ± 44.8	-12.5 ± 37.8	-37.7 ± 24.2	0.23
Glycine	609.8 ± 128.3	18.9 ± 133.8	-22.9 ± 229.4	0.92
Histidine	30.7 ± 9.5	-1.4 ± 9.1	0.1 ± 5.0	0.62
Hydroxy-proline	7.4 ± 1.9	-0.7 ± 1.7[a]	-2.3 ± 0.3[b]	0.06
Isoleucine	97.5 ± 16.5	4.9 ± 14.4	13.0 ± 17.3	0.31
Leucine	107.9 ± 19.0	3.0 ± 17.0	15.5 ± 16.5	0.19
Lysine	73.3 ± 16.3	0.8 ± 20.9	18.3 ± 23.5	0.06
Ornithine	31.3 ± 27.1	0.9 ± 11.0[a]	16.2 ± 13.2[b]	0.06
Phenylalanine	48.7 ± 5.7	-1.6 ± 4.8	4.7 ± 20.1	0.69
Proline	80.6 ± 14.3	-4.2 ± 10.4	-3.8 ± 9.8	0.92
Serine	135.3 ± 76.5	3.1 ± 26.4	-6.9 ± 23.4	0.37
Taurine	27.4 ± 11.1	-2.2 ± 5.4	2.7 ± 5.3	0.16
Threonine	103.4 ± 20.3	-5.1 ± 23.2	1.8 ± 21.5	0.62
Tyrosine	51.9 ± 10.5	-5.7 ± 12.9	-1.2 ± 20.9	1.00
Valine	209.6 ± 28.8	3.5 ± 18.2[a]	29.7 ± 27.4[b]	0.09

TABLE 3: Correlation coefficient between the concentration of amino acid in the plasma and in the follicular fluid on Day 42 post partum for cows classified as clean (<18%PMN) or scEndo resolved (<18%PMN). Bolding of P value indicates significance (P <0.05). %PMN = Percentage of polymorphonuclear neutrophils in the uterus.

Amino acid	Clean		Resolved	
	Coefficient	P value	Coefficient	P value
Number of cows	15		6	
Alanine	0.59	0.02	0.60	0.21
Arginine	0.23	0.42	0.14	0.79
Asparagine	0.67	<0.01	0.83	**0.04**
Aspartate	0.01	0.96	0.60	0.21
Citrulline	0.74	<0.01	0.94	**<0.01**
Glutamate	0.35	0.20	-0.03	0.96
Glutamine	0.54	0.04	0.94	**<0.01**
Glycine	0.96	<0.001	0.94	**<0.01**
Histidine	0.49	0.06	0.49	0.33
Hydroxy-proline	0.84	<0.001	1.00	**<0.001**
Isoleucine	0.70	<0.01	0.66	0.16
Leucine	0.79	<0.01	0.60	0.21
Lysine	0.29	0.29	0.43	0.40
Ornithine	0.58	0.03	0.54	0.27
Phenylalanine	0.45	0.10	-0.03	0.96
Proline	0.60	0.02	0.94	**<0.01**
Serine	0.94	<0.001	0.77	0.07
Taurine	0.90	<0.001	0.26	0.62
Threonine	0.87	<0.001	0.09	0.87
Tyrosine	0.60	0.02	0.77	0.07
Valine	0.81	<0.001	0.66	0.16

cows. In contrast, hydroxy-proline concentrations decreased (P = 0.06) to a greater extent in cows that self-resolved compared with Clean cows.

Correlation of plasma and follicular AA concentrations

In the Clean cows, significant (P <0.1) correlations between blood and follicular AA concentrations were found except for arginine, aspartate, glutamate, lysine and phenylalanine (Table 3). In contrast, a greater number of correlations between blood and follicular AA in cows that self-resolved were non-significant (P >0.1). These amino acids were arginine, glutamate, histidine, lysine, ornithine, phenylalanine, taurine and threonine (Table 3).

DISCUSSION

A subclinical uterine infection such as scEndo at D21 postpartum, and the subsequent self-resolving of this sub-clinical infection was associated with altered follicular AA concentrations. In addition, the follicular AA profile and correlations between plasma and follicular AA were determined in lactating, pasture-fed dairy cows.

In the current study, a large number of the AA determined to be different in the follicular fluid of Clean and scEndo cows have known roles in immune function *e.g.* histidine is important in triggering the inflammation response and can shift fatty acid metabolism away from pro-inflammatory mediators (Li *et al.,* 2007). Other AA (alanine, aspartate, proline, glutamate, valine) have roles in the metabolism and function of cells of the innate immune system (leucocytes, lymphocytes and T-cells; Li *et al.,* 2007) and also as energy substrates (Wu, 2009). Thus, in the current study, differences in AA concentrations appear to indicate follicle function, rather than size; as scEndo had no effect on size, is associated with early postpartum uterine health status. Future work is required to establish if changes in follicular AA concentrations in relation to scEndo are detrimental to the quality of oocyte and thus its fertilisation potential.

In the present study, a more pronounced local effect of scEndo on AA concentrations was evident, as follicular concentrations of histidine, alanine, aspartate and serine were elevated, whereas only aspartate and serine were elevated in plasma from the same animals (Lopdell *et al.,* 2011). In cows that self-resolved, follicular concentrations of aspartate, glutamine, valine and ornithine demonstrated greater changes in concentration, which differed to alterations in plasma, where alanine, asparagine, phenylalanine, serine, threonine, tyrosine and valine concentrations were affected by the infection resolving (Lopdell *et al*., 2011).

To identify if a relationship exists between blood and follicular concentrations, correlation coefficients were determined. The AA with significant correlations suggests a relationship where follicular AA concentrations mirror what is happening in plasma concentrations. The non-significant correlations suggest that for these AA, there is no relationship, and potentially they are being actively excluded from or transported between the follicular environment and plasma (Pelland *et al.,* 2010). These AA are thus of greater potential interest, as it is tempting to postulate that these have a specific biological role within the follicle. It should however be acknowledged that in the present study, while there is a greater number of AA affected in self-resolved cows, the low sample number precludes too much emphasis being placed on the individual AA results. However, overall changes in individual AA concentrations would support the hypothesis that scEndo may be associated with long-term alterations of follicular steroid concentrations. This might ultimately have implications for oocyte viability, embryo

development and subsequent survival, since AA metabolism can affect the ability of bovine embryos to develop (Sturmey *et al.,* 2010).

This study established a follicular AA profile for lactating, pasture-fed dairy cows, of which there is limited published information. The AA profiles determined for pasture-fed cows in the current study differ considerably from those of overseas concentrate-fed cows (Orsi *et al.,* 2005). Concentrations of most follicular AA measured were lower in pasture-fed than concentrate-fed cows, although large variation exists in the amount these AA varied in relation to concentrate-fed cows. Generally, AA ranged from those that were 25-50% lower such as aspartate, glutamine, tyrosine, valine and isoleucine; those that were 50-100% lower such as glutamine, alanine, phenylalanine, leucine and lysine; and those that over 100% lower such as histidine, threonine, arginine and taurine. In contrast, the concentration of several AA, such as asparagine, serine and glycine, were substantially greater in pasture fed than concentrate-fed cows. While is it beyond the scope of this paper to speculate what these differences may mean, there was a consistent trend across both studies for the most abundant AA in follicular fluid to be glutamine, glycine, alanine and valine.

In conclusion, it appears that sub-clinical uterine infection is associated with long-lasting alterations in follicular AA concentrations. Whether altered follicular AA concentrations have a negative impact on oocyte viability and ultimately the sub-fertility associated with uterine infection remains to be determined.

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