Effect of resolving a sub-clinical uterine infection on plasma and follicular fluid steroid concentrations in early lactation dairy cows

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Abstract

Sub-clinical endometritis (scEndo) in early lactation affects ovarian follicle dynamics, delays a return to cyclicity, with changes in plasma and follicular fluid (FF) amino acid concentrations around the time of rebreeding. This study examined the effect of resolving scEndo in early lactation on plasma and FF oestrogen, progestagen, androgen and corticosteroid concentrations at the time of rebreeding in 46 dairy cattle. On D42 postpartum, cows were classified as having scEndo, clean or having resolved scEndo based on uterine cytology on D21 of > 18% polymorphonuclear (PMN) cells amongst uterine nucleated cells. On D42 and D63 follicular and plasma samples were collected and steroid concentrations analysed. On D21 and D42, 35% and 7% of cows respectively were classified as having scEndo, with 81% of scEndo cows having self-resolved the infection by D42. In those cows that resolved scEndo, none of the plasma or FF steroid concentrations changed between D42 and D63, except for plasma progesterone (P = 0.08). Using a low (4%PMN) threshold to classify scEndo did not identify any significant changes in steroid concentrations. In contrast to previously described changes in plasma and FF amino acid concentrations, there were no significant effects on steroid concentrations when cows self-resolved a sub-clinical uterine infection.

Keywords: steroid; plasma; follicular fluid; 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 (Evans & Walsh 2012). Endometritis in dairy cows is caused by bacterial infection and leads to inflammation of the uterine endometrium (Kasimanickam et al. 2004). It is associated with reproductive problems, such as lower conception rates and longer intervals between calving and conception (Williams et al. 2005) and is estimated to affect approximately 15% of cows (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 is difficult to detect due to the lack of obvious symptoms, so its impact on reproduction is poorly understood (Le Blanc 2008).

Both clinical and sub-clinical uterine infection impairs uterine function and disrupts ovarian cyclicity (Williams et al. 2007). Consequences of these infections include decreased follicular growth and oestradiol production (Herath et al. 2007), and reduced corpus luteum (CL) size and function (Williams et al. 2007). Furthermore, Lavon et al. (2011) reported abnormal follicular steroidogenesis and decreased steroid gene expression, which may have contributed to lower follicular steroid production, in bovine pre-ovulatory follicles. Hence uterine and mammary bacterial infection has the potential to influence ovulation, conception and pregnancy success.

Previously we demonstrated that scEndo has wide-ranging effects in pasture-fed cows during early lactation. This included altered haematological parameters and plasma albumin concentrations, lowered milk fat and protein concentrations (Green et al. 2009). It also has the ability to perturb nutrient supply as demonstrated by long-term alterations in follicular and plasma AA concentrations (Back et al. 2011; Lopdell et al. 2011). In addition it is associated with a delayed return to cyclicity, with alterations in follicular steroid hormone concentrations (Green et al. 2011).

The objective of the present study was to examine the effects in early lactation of self-resolving scEndo on the plasma and follicular concentrations of steroid hormones around the standard time of rebreeding between 42 and 63 days postpartum. It is postulated that cows that self-resolve scEndo may exhibit altered, potentially sub-optimal, steroid hormone concentrations during this critical period.

Material and methods

This study was approved by the Ruakura Animal Ethics Committee and conducted from August to October. 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 46 mixed-age lactating dairy cows was assessed by determination of percentage of polymorphonuclear neutrophils (%PMN) of the uterine endometrium. Uterine endometrial samples were taken on Day (D) 21 and D42 postpartum (± 3 days) for cytological analysis using a cytobrush technique (Kasimanickam et al. 2005) 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 into heparinised vacutainers (Becton Dickenson, Auckland). Samples were centrifuged for 15 minutes at 1500 *g* at 4ºC and the resulting plasma stored at -20°C until analysis.

Follicular and luteal dynamics were evaluated by ultrasonography as described by Green et al. (2011). 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 and four days later the diameter of the new lead follicle was measured. Lead follicles were defined as the largest follicle on either ovary at assessment that eventually would have become the pre-ovulatory dominant follicle. 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 steroid analysis. Plasma and follicular fluid concentrations of nine steroids were measured in individual samples using High Performance Liquid Chromatography (HPLC) (Green et al. 2011). Due to low concentrations plasma oestradiol was measured by radioimmunoassay (Siemens Healthcare Diagnostics Ltd., Auckland, New Zealand) following the manufacturer's instructions.

Statistical analyses

Differences between follicle size with respect to day postpartum and uterine status were analysed as previously described (Green et al. 2011). For follicular fluid steroid data, only those cows that provided uncontaminated samples on D42 and D63 postpartum were used in the analysis.

The steroid hormone data were analysed using two methods. Firstly, to test for an effect of day postpartum on plasma and FF steroid concentration the PROC MIXED function with Tukey's *post-hoc* analysis (SAS 2006) was employed. Cows were assigned their uterine status as being 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 and follicular size as a covariate.

Secondly, the effect of uterine status or day postpartum on changes in FF steroid concentrations of cows in which sEndo self-resolved, that is they changed their uterine status on D21 from scEndo to clean on D42 postpartum, were investigated. Cows were classified as Clean if they were clean on both days, scEndo if they were scEndo on both days, or Resolved if scEndo on D21 and clean on D42, and the change in medians compared with the self-resolved group were determined. These data were analysed using the Kruskal-Wallis (KW) rank sum test in R version 2.14 (R Core Development Team 2011). In addition, since debate exists in the literature on the most relevant %PMN threshold to use to define scEndo, the data were analysed and compared using both 18 %PMN (Kasimanickam et al. 2004) and 4 %PMN (Dubuc et al., 2010). The latter was the median of the %PMN for D21 and D42 combined, in the current study.

Results

Prevalence of scEndo

Uterine cytological examinations of 46 D21 cows 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%).

Collection of follicular samples and luteal status

Follicles were smaller ($P < 0.001$) on D42 than on D63 postpartum (9.5 ± 0.36 mm and 11.5 ± 0.38 mm respectively), although no difference was evident with respect to uterine status. Not all cows had lead follicles large enough to sample, of those that did, uncontaminated follicular fluid samples were successfully collected from 31 of 45 cows on D42 and from 35 of 46 cows on D63. In the first analysis (Table 1), only data from Clean (n = 28) and Resolved (n = 32). In the second analysis (Table 2), 27 cows provided uncontaminated follicular samples on both days. Of these only 24 cows maintained the same uterine status classification on D42 and D63 and were included. The percentage of cows determined, via the presence of corpora lutea, to have returned to cyclicity on D21, D42 and D63 were 43%, 82% and 91% respectively. Cows classified as having scEndo using both a 4 and 18% threshold, demonstrated a slower return to cyclicity (Green et al. 2011).

Table 1 Mean ± standard error of steroid concentrations (ng/mL) in plasma and follicular fluid of dairy cows on Day 42 and Day 63 postpartum. P value in italics indicates significance between P = 0.05 and P = 0.10. DHEA = Dehydroepiandrosterone; ND = Not detected.

Tissue	Steroid	Day 42	Day 63	Range	P value
Plasma	Number of samples	39	42		
	Androstenedione	ND	ND	ND	
	Cortisol	13.1 ± 1.4	13.2 ± 1.5	1.7–40.0	0.93
	Corticosterone	0.6 ± 0.1	0.6 ± 0.1	0.1–2.2	0.89
	11-deoxycortisol	0.3 ± 0.1	0.3 ± 0.1	0.1–1.2	0.50
	DHEA	0.40 ± 0.04	0.40 ± 0.03	0.1–1.0	0.73
	Oestradiol	0.004 ± 0.001	0.003 ± 0.001	0.001–0.01	0.16
	Oestrone	ND	ND	ND	
	Progesterone	3.6 ± 0.5	4.5 ± 0.5	0.1–11.3	*0.08*
	Testosterone	ND	ND	ND	
Follicular fluid	Number of samples	28	32		
	Androstenedione	19.4 ± 5.6	20.3 ± 4.8	0.1–151.2	0.90
	Cortisol	4.6 ± 0.4	4.4 ± 0.3	1.7–11.5	0.52
	Corticosterone	ND	ND	ND	
	11-deoxycortisol	4.0 ± 0.5	4.6 ± 0.4	0.7–10.4	0.29
	DHEA	3.4 ± 1.0	4.1 ± 0.8	0.4–15.3	0.61
	Oestradiol	248 ± 53	345 ± 48	15–1,235	0.18
	Oestrone	14.8 ± 6.0	14.8 ± 5.2	0.1–160.0	0.99
	Progesterone	34 ± 8	44 ± 8	11–1,068	0.41
	Testosterone	3.7 ± 0.8	2.9 ± 0.7	0.7–23.1	0.44

Measurement of plasma and follicular steroids

Plasma samples were collected from 42 of 45 cows on D42 and from 41 of 46 cows on D63. The concentration of five of the eight steroids in plasma and eight of the nine steroids in follicular fluid equivalent to more than 80% of the samples, were successfully measured by HPLC analysis. The remainder were below the detection limit (Table 1).

Changes in steroid concentration

The follicular concentration of the majority of steroids, especially the oestrogens, was substantially higher than in plasma. Cortisol and corticosterone concentrations were the exception, as these were higher in plasma than follicular fluid (Table 1). Only plasma progesterone concentration tended (P = 0.08) to increase between D42 and D63. No differences between days in any of the other steroid concentrations were observed in plasma or FF.

Using an 18%PMN threshold to classify scEndo, no differences (P < 0.05) were identified in mean FF steroid concentrations between the Clean, scEndo and Resolved groups or in changes over time in median FF concentrations of resolving cows (TJ Lopdell, Unpublished data). With a 4% PMN threshold, significant differences (P < 0.05) were evident to mean steroid concentrations with respect to uterine status (Table 2). On D42 mean cortisol and on D63 mean oestrone concentrations were higher in the FF of those cows that had resolved an infection compared with those than were clean. Those cows classified as having scEndo at these time points had concentrations in between the other two groups. In addition, there tended (P < 0.1) to be changes in the median concentrations of cortisol on D42 (P = 0.08) and oestrone on D63 (P = 0.09) for cows that resolved a uterine infection.

Discussion

The current study found that firstly, FF steroid concentrations were generally far higher than those in plasma of postpartum cows, secondly that the PMN% threshold of 18% versus 4% chosen to classify scEndo, can affect whether significant differences in mean FF steroid concentrations were identified, with none evident using 18%PMN, and thirdly that cows in which a uterine infection self-resolved before D42, only tended to demonstrate changes in FF cortisol and oestrone concentrations.

Concentrations of most steroids were higher in FF than in plasma on both D42 and D63, as would be expected, since the follicle is the principle site of steroid production in the postpartum cow. Exceptions were higher plasma cortisol concentrations, and the detection of corticosterone which was not detected in FF. Both these steroids are glucocorticoids, synthesised predominantly by the adrenal glands as part of the stress response (Breen & Karsch 2006). Therefore higher concentrations in circulation maybe due to a systemic response to the sampling regimen, rather than an increased local ovarian production. Although it should be acknowledged that plasma cortisol concentrations are linked with infection severity in other diseases (Christ-Crain et al. 2007) and cortisol is postulated to increase the susceptibility of the uterus to infection (Torres et al. 2007). Differences over time, rather than between follicular and

Table 2 Mean ± standard error of Day 42 and Day 63 steroid concentrations (ng/mL) in follicular fluid of dairy cows with uterine status classified as Clean (<4 percent of polymorphonuclear neutrophils in the uterus (%PMN); n = 12) and scEndo (>4% PMN; n = 3) based on Day 21 and Day 42 postpartum %PMN results. Plus concentrations for cows classified as having a Resolved (D21 scEndo and Day 42 Clean; n = 9) uterine status. P value shows the KW test for equality of medians (TJ Lopdell, Unpublished data) between the Resolved and the other two groups. Superscripts within rows indicate a difference (P < 0.05) between mean concentrations between the three groups. P value in italics indicates significance between P = 0.05 and P = 0.10. DHEA = Dehydroepiandrosterone; ND = Not detected.

Days postpartum	Steroid	Clean	scEndo	Resolved	Change in median
					P value
42	Number of follicles	12	3	9	
	Androstenedione	26 ± 13	37 ± 18	20 ± 5	0.47
	Cortisol	3.7 ± 0.5[a]	4.4 ± 0.4[ab]	5.1 ± 0.5[b]	*0.08*
	Corticosterone	ND	ND	ND	
	11-deoxycortisol	4.6 ± 0.8	3.5 ± 1.4	3.9 ± 0.7	0.75
	DHEA	4.2 ± 1.6	4.3 ± 0.5	3.5 ± 1.2	0.86
	Oestradiol	319 ± 70	339 ± 166	249 ± 70	0.69
	Oestrone	21 ± 13	27 ± 15	15 ± 5	0.41
	Progesterone	28 ± 4	75 ± 56	50 ± 19	0.97
	Testosterone	4.6 ± 1.9	2.5± 1.6	3.8 ± 0.8	0.75
63	Number of follicles	12	3	9	
	Androstenedione	17 ± 7	19 ± 18	19 ± 6	0.67
	Cortisol	3.9 ± 0.5	4.0 ± 0.3	4.7 ± 0.7	0.17
	Corticosterone	ND	ND	ND	
	11-deoxycortisol	4.3 ± 0.6	4.4 ± 2.4	4.5 ± 0.8	0.94
	DHEA	2.0 ± 0.6	4.0 ± 3.1	3.7 ± 0.8	0.21
	Oestradiol	272 ± 66	353 ± 251	329 ± 84	0.40
	Oestrone	5.8 ± 1.6[a]	11.2 ± 5.9[ab]	18.2 ± 5.2[b]	*0.09*
	Progesterone	30 ± 4	24 ±.8	50 ± 28	0.46
	Testosterone	2.8 ± 0.5	2.0 ± 1.3	3.2 ± 0.7	0.74

circulating concentrations, were only identified in plasma progesterone where the concentration tended to increase, most likely due to an increasing number of cows returning to a normal oestrous cycle. In addition, the first oestrous cycle after calving is known to result in lower circulating progesterone concentrations than later cycles (Pope et al. 1969).

Differences in follicular steroid concentrations associated with uterine status, were found in the current study when a 4%PMN threshold was used. On D42 mean follicular cortisol concentration was lower in Clean cows than in cows that had resolved an infection. This may be because cortisol metabolism within the follicle was compromised due to the resolving of an infection in these cows. Similarly on D63 this may also explain the increased follicular oestrone concentration in cows that had resolved an infection. The reason why scEndo cows, despite similar elevated follicular cortisol and oestrone concentrations, were not significantly different from Clean cows is likely to be due to low number of cows in the group. The authors postulate that with increased numbers significance would have been achieved.

This study also investigated the implications of using two PMN% thresholds to define scEndo, as there has been debate in the literature over when to sample and the appropriate threshold at different postpartum intervals (Lopdell et al. 2011). The prevalence of scEndo in this study (7% at D42) using a PMN% threshold of 18%, is in line with results from other studies, such as Dubuc et al. (2010), who had 13.9% at D35, and Kaufmann et al. (2009), who had 13.4% scEndo at first service with a PMN% threshold of 15%. When using the lower 4% PMN% threshold, the current study had a scEndo prevalence of 23%, which is in agreement with Dubuc et al. (2010) who reported a prevalence of 21.1%.

It must be acknowledged that the small sample size in this study is a limitation, and that because of the high self-resolve rates in the scEndo cows from D21 to D42, only six cows at a 4% threshold and three cows at 18% had scEndo by D42. Therefore changes in steroid concentrations, particularly in the scEndo cows should be treated with caution. A larger study is needed to confirm any differences, should they exist.

In conclusion, there appears to be little effect of self-resolving scEndo on the plasma and follicular concentrations of steroid hormones around the time of rebreeding on D42 and D63 postpartum in pasture-fed dairy cows. Based on this finding, in contrast to those cows with scEndo or a clinical infection (Le Blanc, 2008), it is unlikely that the rebreeding potential of

these cows would be affected. However, the small sample size in this study would support further investigation of the long-term fertility implications of resolving a sub-clinical uterine infection.

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