

Influence of energy balance on the antimicrobial peptides S100A8 and S100A9 in the endometrium of the *post-partum* dairy cow

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Abstract

Uterine inflammation occurs after calving in association with extensive endometrial remodelling and bacterial contamination. If the inflammation persists, it leads to reduced fertility. Chronic endometritis is highly prevalent in high-yielding cows that experience negative energy balance (NEB) in early lactation. This study investigated the effect of NEB on the antimicrobial peptides S100A8 and S100A9 in involuting uteri collected 2 weeks *post partum*. Holstein-Friesian cows (six per treatment) were randomly allocated to two interventions designed to produce mild or severe NEB (MNEB and SNEB) status. Endometrial samples were examined histologically, and the presence of neutrophils, macrophages, lymphocytes and natural killer cells was confirmed using haematoxylin and eosin and immunostaining. SNEB cows had greater signs of uterine inflammation. Samples of previously gravid uterine horn were used to localise S100A8 and S100A9 by immunohistochemistry. Both S100 proteins were present in bovine endometrium with strong staining in epithelial and stromal cells and in infiltrated leucocytes. Immunostaining was significantly higher in SNEB cows along with increased numbers of segmented neutrophils. These results suggest that the metabolic changes of a *post-partum* cow suffering from NEB delay uterine involution and promote a chronic state of inflammation. We show that upregulation of S100A8 and S100A9 is clearly a key component of the early endometrial response to uterine infection. Further studies are warranted to link the extent of this response after calving to the likelihood of cows developing endometritis and to their subsequent fertility.

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Introduction

The *post-partum* period is critical for subsequent fertility in dairy cows, as the resumption of ovarian cyclicity and uterine recovery are both essential before conception can occur again (Sheldon 2004). Uterine bacterial contamination is almost universal after calving (Foldi *et al.* 2006). In most cows, this is eliminated during the process of involution, but cows that fail to eliminate potentially pathogenic microorganisms can develop metritis or endometritis (Sheldon 2004, Chapwanya *et al.* 2009). These diseases are highly prevalent in high-yielding cows (LeBlanc *et al.* 2002, Gilbert *et al.* 2005) and are associated with an inflammatory response. Endometritis was described by Bondurant (1999) as a superficial inflammation of the endometrium extending no deeper than the stromal layer, with some disruption of the surface epithelium, infiltration with

inflammatory cells (neutrophils, macrophages, eosinophils, mast cells, lymphocytes and plasma cells), vascular congestion and stromal oedema. An increase in segmented neutrophils (or polymorphonuclear leucocytes, PMNs) in the endometrium was associated with poor reproductive performance, whereas the presence of lymphocytic foci in endometrium was associated with good fertility (Bonnett *et al.* 1993). Endometritis causes economic losses through increases in calving interval, a higher culling rate for infertility, and reduced milk yield (Dobson & Smith 1998, Gilbert *et al.* 2005).

The endometrium is the first line of defence against invading microorganisms. Rapid defence mechanisms are provided by innate immunity, which recognises pathogens mainly by the family of toll-like receptors (TLRs; Werling & Jungi 2003). Among the TLR family members, TLR4, found in bovine endometrial epithelial

and stromal cells, is capable of recognising lipopolysaccharide (LPS) from Gram-negative bacteria including *Escherichia coli* (Herath *et al.* 2006). LPS binds to MD2 (an LPS-binding protein) and interacts with CD14 (the main LPS receptor) and then with TLR4, thus activating a signal transduction pathway in cells. This leads to the production of a number of cytokines and chemokines (Chapwanya *et al.* 2009, Fischer *et al.* 2010, Galvao *et al.* 2011), antimicrobial peptides (AMPs), acute phase proteins (APPs) (Davies *et al.* 2008, Chapwanya *et al.* 2012) and prostaglandins (Herath *et al.* 2009a). We have previously shown that several AMPs involved in innate immunity, including both defensins and calcium-binding proteins S100, are expressed in bovine endometrium (Wathes *et al.* 2009).

S100A8 and S100A9 are calcium-binding proteins that belong to the S100 protein family (Kligman & Hilt 1988). They have previously been reported to have a variety of actions in innate immunity (Vandal *et al.* 2003), as an antimicrobial (Sohnle *et al.* 2000), in wound healing (Thorey *et al.* 2001) and they also act as a survival factor for early-stage embryos (Passey *et al.* 1999). S100A8 and S100A9 are mainly found as cytosolic protein in neutrophils and monocytes (Ahmad *et al.* 2003) but they have also been detected in other cell types and tissues such as leukocytes, fibroblasts, tumour cell lines and bone marrow (Dale *et al.* 1985, Rahimi *et al.* 2005) including both squamous epithelia and non-squamous cell types (Matsumoto *et al.* 1997). They were also present in the human reproductive tract (myometrium and cervix) around parturition (Havelock *et al.* 2005). We recently demonstrated that LPS caused a rapid upregulation of *S100A8*, *S100A9* and *S100A12* mRNAs and S100A8 protein in cultured bovine endometrium (Swangchan-Uthai *et al.* 2012).

Genetic selection of dairy cows for high milk yields has been associated with a reduction in fertility (Veerkamp *et al.* 2001). Modern dairy cows are often unable to meet their energy requirements for body maintenance and milk production and then enter a stage of negative energy balance (NEB). This is associated with excessive lipid metabolism and loss of body condition score (BCS), resulting in metabolic disorders via alterations in blood metabolites and subsequently reduced fertility (Roche *et al.* 2000, Butler 2003, Wathes *et al.* 2007). During the nadir of NEB, the limitations on nutrient availability also impair immune function such as neutrophil activity (Hammon *et al.* 2006). This in turn appears to increase the susceptibility to infections such as endometritis and mastitis (Wathes 2012).

By developing an energy balance model to provide two groups of cows with induced mild or severe NEB (MNEB and SNEB) status in early lactation, we previously demonstrated that NEB can affect many systems in the body that contribute to successful pregnancy. This included effects on the liver, spleen, ovary and oviduct as well as the uterine environment (reviewed by Wathes

et al. (2008) and Wathes (2012)). As the genes encoding S100A8 and S100A9 were highly upregulated in the endometrium of SNEB cows (Wathes *et al.* 2009), we speculated that these proteins may act as an indicator of the severity of endometrial inflammation after calving. This idea was supported by a recent review of the literature concerning the role of the S100 family in obstetrics and gynaecology, which reported associations between the amounts of S100A8 and S100A9 in endometrial and cervical cancer respectively (Kostakis *et al.* 2010). However, the localisation of these proteins in the endometrium and the factors influencing their production *in vivo* has not, to our knowledge, been investigated previously. In this study, we have examined the presence of S100A8 and S100A9 in bovine endometrium and their relationship with metabolic status and endometrial inflammation in *post-partum* dairy cows experiencing different degrees of NEB. Our hypothesis is that the metabolic changes associated with SNEB delays uterine clearance mechanisms and promotes a chronic state of inflammation that can detrimentally affect fertility.

Results

Energy balance and hormone and metabolite measurements collected from a cohort of 12 multiparous Holstein-Friesian dairy cows managed to achieve a state of MNEB or SNEB after calving has been reported in detail previously (Fenwick *et al.* 2008). In summary, the net energy values on the day of killing for tissue sample collection (13.6 ± 0.8 and 14.3 ± 0.6 days respectively) were about threefold lower in the SNEB than in the MNEB cows (-2.7 ± 1.41 vs -6.1 ± 1.03 unite four-ragère lait (UFL)/day). The SNEB cows had clear evidence of fatty liver, associated with lipid infiltration. Circulating concentrations of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) were elevated in SNEB and IGF1 and glucose levels were significantly decreased. All SNEB cows were thus in subclinical ketosis (≥ 1.2 mmol/l) with plasma BHB concentrations ranging from 3.0 to 4.5 mmol/l.

Immune cell infiltration into the endometrium

Histological analysis based on haematoxylin and eosin (H&E) staining was performed separately for the previously gravid and non-gravid uterine horns (with diameters of 56 ± 7 vs 31 ± 3 mm respectively, values are mean \pm S.E.M.) and in each horn separately for the caruncular and intercaruncular regions. Segmented cells representing neutrophils were found in the luminal and glandular epithelium and the stroma in all sections examined (Fig. 1A). Mononuclear cells representing lymphocytes were found as lymphocytic foci in the stroma and in the basement of the luminal epithelium (Fig. 1B). The number of segmented neutrophils in both

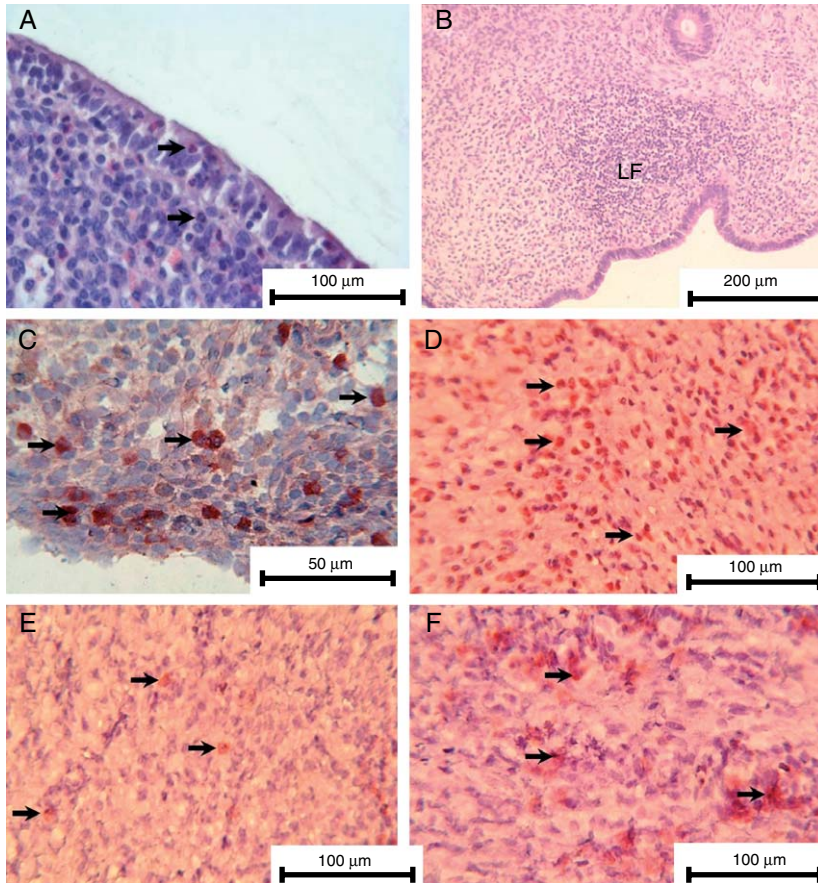


Figure 1 Representative photomicrographs of *post-partum* bovine endometrium stained with H&E (A and B) and using immunohistochemistry for CD172A (a macrophage and granulocyte marker, C); CD14 (a macrophage marker, D); CD2 (a T-cell and NK cell marker, E) and CD8 (a cytotoxic T-cell marker, F). In A, the arrows indicate segmented neutrophils in both luminal epithelium and stroma. B shows a lymphocytic focus (LF) in the stroma. The arrows in C, D, E, and F indicate positive immunostaining of immune cells.

luminal epithelium and stroma was significantly higher in SNEB cows than in MNEB cows (77.8 ± 20.4 vs 22.4 ± 6.6 cells per graticule, $P < 0.05$; 9.2 ± 1.3 vs 3.8 ± 0.83 cells per $10 \mu\text{m}^2$, $P < 0.01$ respectively). More stromal lymphocytic foci per section were also detected in SNEB cows (6.2 ± 1.0 vs 2.0 ± 0.5 , $P < 0.001$). Mean macrophage numbers in endometrial tissues were not different between the SNEB and the MNEB groups. SNEB cows also had more capillaries (7.2 ± 0.6 vs 5.1 ± 0.4 and 7.4 ± 0.4 vs 5.8 ± 0.5 per $20 \mu\text{m}^2$ in gravid horn and non-gravid horns respectively, both $P < 0.05$).

Immunohistochemistry was used to confirm the identity of the immune cell types present in the endometrium. Positive immunostaining was detected for tyrosine protein phosphatase non-receptor type substrate 1 (CD172A, representing macrophages and granulocytes, Fig. 1C), CD14 cell surface antigen (a macrophage marker; Fig. 1D), CD2 molecule (T-cell and natural killer (NK) cell marker; Fig. 1E) and T-cell surface glycoprotein CD8 (cytotoxic T-cell marker; Fig. 1F).

S100A8 and S100A9 in the endometrium

Positive immunostaining for both S100A8 and S100A9 was noted in the endometrial epithelial and stromal

layers of both intercaruncular and caruncular regions from all 12 cows (Fig. 2A, B, C, D, E, and F). Liver was used as a positive control tissue (Fig. 2G and H), whereas neither S100A8 nor S100A9 was detectable in the ovarian tissues examined (Fig. 2J and K). The isotype IgG-negative controls are also shown in Fig. 2C, F, I, and L.

Localisation of S100A8 and S100A9 within different endometrial regions of interest (ROI) is illustrated in Fig. 3. The levels of immunostaining varied between cows, but in general, there was strong or moderate positive staining in luminal epithelium and infiltrated leukocytes in both luminal epithelium and stroma, whereas staining in the glandular epithelium was weak or absent. Mixed model analysis was next used to compare staining patterns of the protein S100A8 and S100A9 assessed from H-score measurements in the endometrium according to i) NEB status (MNEB and SNEB) and ii) ROIs. Overall levels of S100A8 and S100A9 in the SNEB group were higher when compared with the MNEB group (Fig. 4A and B). S100A8 and S100A9 immunostaining was relatively higher in the luminal epithelium and stroma of both caruncular and intercaruncular endometrium compared with the intercaruncular glandular epithelium. In addition, the

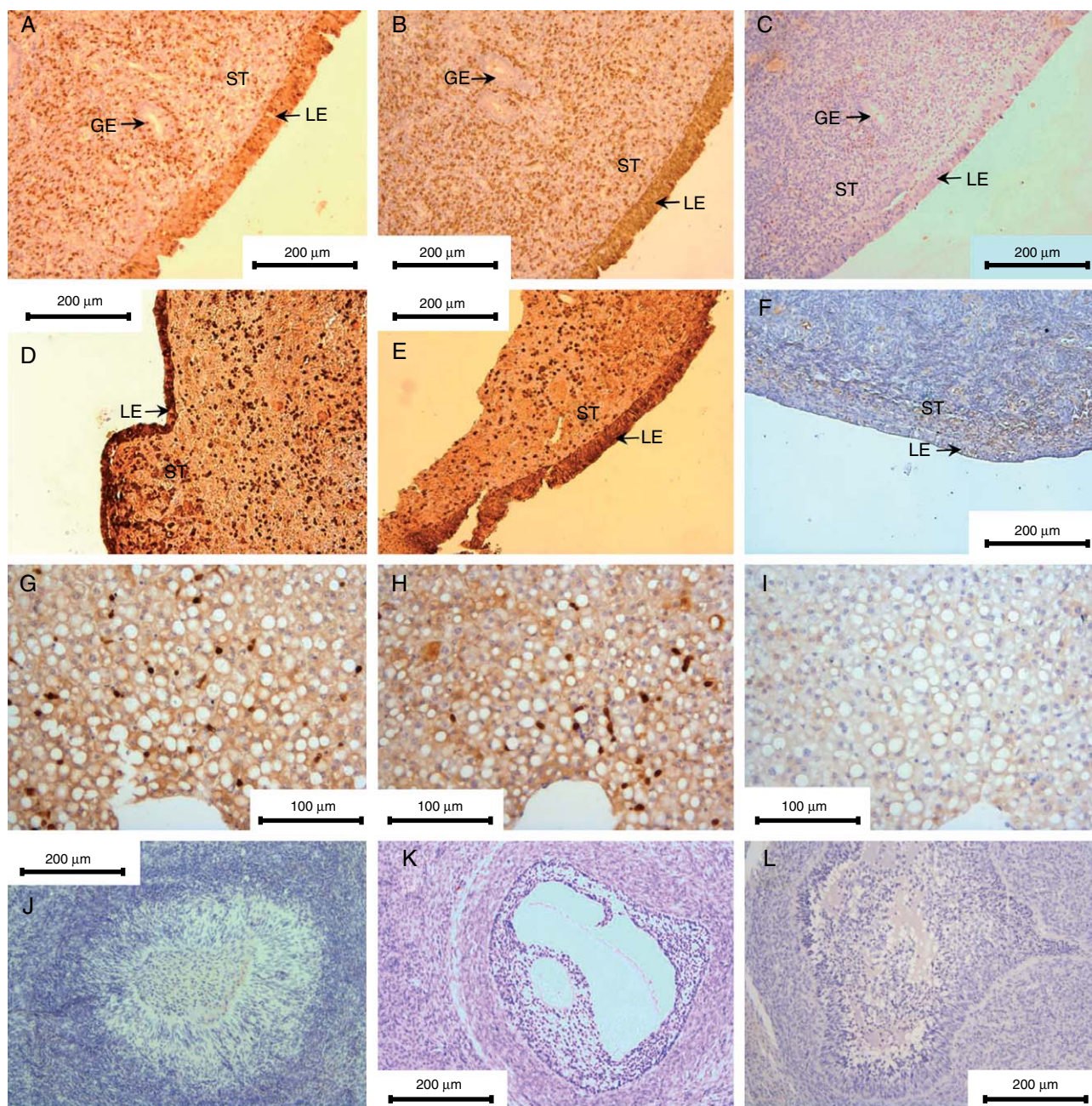


Figure 2 Representative photomicrographs of *post-partum* bovine endometrium showing immunostaining for S100A8 (A, D, G, and J), S100A9 (B, E, H, and K) and isotype IgG-negative control (C, F, I, and L) in the intercaruncular (A, B, and C) and caruncular regions (D, E, and F). Liver (G, H, and I) was used as a positive control tissue and ovary (J, K, and L) as a negative control tissue. The sections were counterstained with haematoxylin. LE, luminal epithelium; GE, glandular epithelium; ST, stroma.

luminal epithelium of both caruncular and intercaruncular endometrium expressed S100A9 protein more highly than the caruncular stroma (Fig. 4C and D). Effects of NEB group were mainly significant in the luminal epithelium, although the H-scores were consistently numerically higher in the SNEB cows for all ROIs. These differences in immunostaining patterns between the MNEB and SNEB groups are further illustrated in Fig. 5, in which one representative cow from each EB group is compared.

Correlation analysis

Pearson correlations, corrected for false discovery rate, were performed to suggest relationships between the H-score values for endometrial S100A8 and S100A9 with other factors, using data across all cows ($n=12$). S100A8 protein levels in each ROI were positively correlated with those of S100A9 in the same region (Table 1), showing that both proteins were upregulated together.

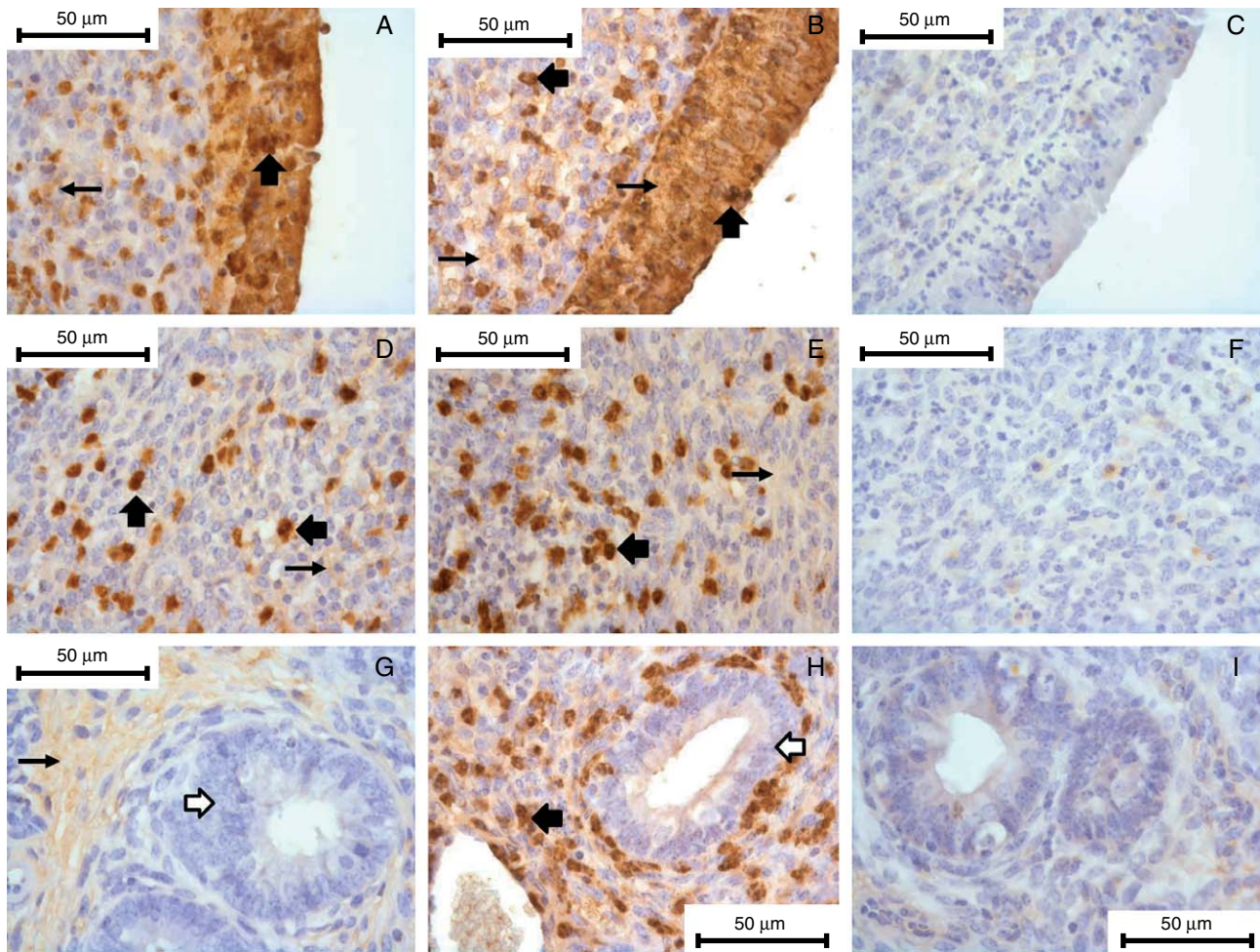


Figure 3 Representative photomicrographs of *post-partum* bovine endometrium in the intercaruncular region showing immunostaining for S100A8 (A, D, and G), S100A9 (B, E, and H) and isotype-negative control (C, F, and I) showing (A, B, and C) luminal epithelium, (D, E, and F) stroma and (G, H, and I) glandular epithelium. Strong positive staining in luminal epithelium and infiltrated leukocytes in both luminal epithelium and stroma is indicated by large closed arrows. Moderate staining (small arrows) in stroma and negative to weak staining (open arrows) in glandular epithelium can also be noted.

The *S100A8* mRNA concentrations measured by both qPCR and from the microarray were correlated with the S100A8 immunostaining levels (Table 2). The mRNA levels of *S100A8*, *S100A9* and *S100A12* from the microarray data were consistently positively correlated with the immunostaining levels of S100A8 and S100A9 in the luminal epithelium of both intercaruncular and caruncular tissue ($P < 0.05$, Table 2). There were also some significant relationships with protein levels in the caruncular stroma and glandular epithelium. Significant correlations were also found between S100A8 and S100A9 protein levels with the concentration of *S100A12* mRNA. In contrast, there were no significant correlations between the immunostaining results and mRNA levels of another S100 family member present on the microarray, *S100A2* (Table 2).

The levels of immunostaining were also related to the number of immune cells counted in the endometrium from the H&E-stained sections (Table 2). The number of

segmented neutrophils infiltrated in the epithelium was correlated with S100A8 and S100A9 immunostaining H-score in the glandular epithelium, caruncular epithelium and caruncular stroma, while the number of segmented neutrophils infiltrated in the stroma correlated with protein levels in the intercaruncular and caruncular luminal epithelium. No significant relationships were detected with either the number of monocytes in the epithelium or stroma or the number of lymphocytic foci in the stroma (data not shown).

Discussion

This study was conducted to investigate immunostaining patterns of the AMPs S100A8 and S100A9 during the *post-partum* period of dairy cattle. The specimens were collected ~2 weeks after calving. At this stage, the caruncle is expected to be undergoing degeneration and sloughing after placental separation and re-epithelisation

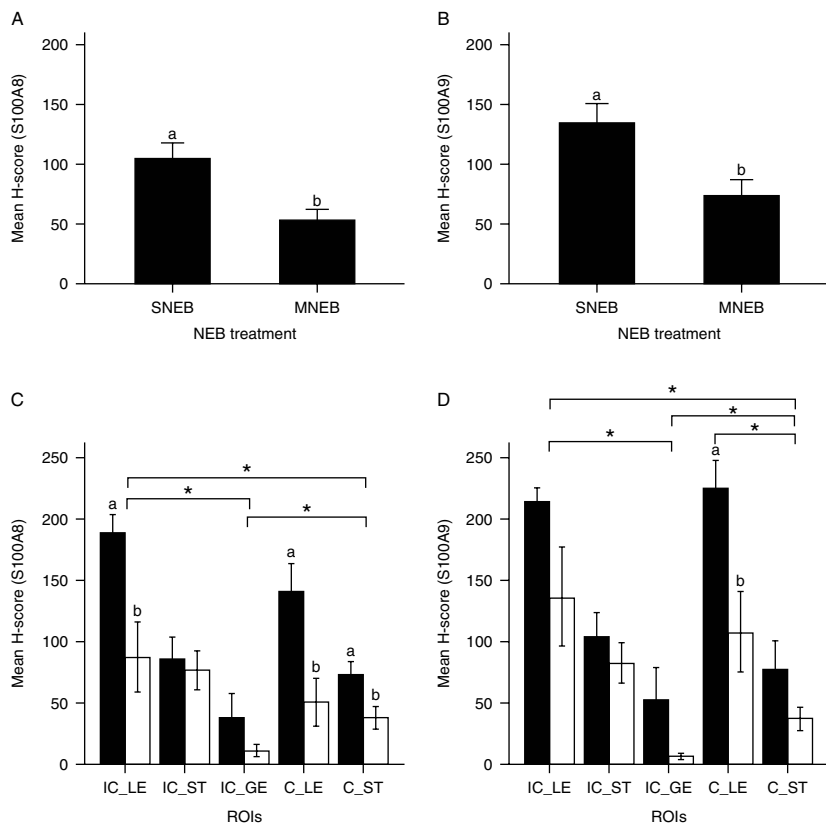


Figure 4 The overall expression (mean H-score \pm S.E.M.) of (A) S100A8 and (B) S100A9 in bovine endometrium according to treatment group (severe or mild negative energy balance, SNEB and MNEB respectively), $n=6$ cows per group: $a>b$, $P<0.05$. H-score \pm S.E.M. of (C) S100A8 and (D) S100A9 in different regions of interest (ROIs) compared between SNEB and MNEB cows (solid and open bars respectively): IC_LE, intercaruncular luminal epithelium; IC_ST, intercaruncular stroma; IC_GE, intercaruncular glandular epithelium; C_LE, caruncular luminal epithelium; C_ST, caruncular stroma. Different superscript letters indicate significant differences between treatment groups ($a>b$), whereas asterisks (*) indicate differences between ROIs ($P<0.05$).

of the endometrium has not yet been completed (Gier & Marion 1968). Both epithelial and stromal cells are commonly contaminated with microbes that produce endotoxins, before self-clearance, which should occur within 6 weeks *post partum* (Griffin *et al.* 1974). High-yielding cows can take up to 20 weeks *post partum* to regain a positive energy balance status (Taylor *et al.* 2003). Thus, the endometrial samples used in this study were collected from animals suffering to various degrees of NEB. They were taken from cows that had not yet resumed oestrous cycles after calving to avoid a potential effect of differential stage of the cycle on the specimens.

Drackley (1999) proposed that inflammation was a missing link in dairy cows between systemic and local uterine pathology as a consequence of metabolic disorders in the transition period (between 3 weeks before and 3 weeks after calving). Many lines of evidence have since supported this relationship. For example, experimentally induced fatty liver in cows resulted in the activation of circulatory TNF α activities and increased levels of the APPs haptoglobin and serum amyloid A (SAA; Ohtsuka *et al.* 2001, Ametaj *et al.* 2009). Using gene microarrays, 103 of the 197 recognised differentially expressed genes in endometrium of SNEB compared with MNEB cows were associated with immune or inflammatory responses (Wathes *et al.* 2009). Recently, Chapwanya *et al.*

(2012) found an increase in endometrial inflammation and leukocyte infiltration together with increased mRNA levels of pro-inflammatory AMP and APP genes during the early *post-partum* period in cows. Two studies that examined endometrial biopsies obtained during the *post-partum* period revealed that mRNA levels of *TLR4*; the pro-inflammatory cytokines *TNF*, *IL6*, *IL1A* and *IL1B*; the receptor *IL1R2*; and the chemokines *CXCL5* and *IL8* were higher in infertile cows with persistent endometritis than in cows with no clinical signs or subclinical endometritis (Herath *et al.* 2009b, Fischer *et al.* 2010). Our histological study of uterine specimens in the energy balance model reported here also provides strong evidence for an ongoing inflammatory reaction with more segmented neutrophils in the endometrium and oedema at 2 weeks *post partum* when cows were in SNEB.

In cyclic and early pregnant cows, T and B lymphocytes were present in the sub-epithelial stroma but they were rarely found within the luminal epithelium or the uterine lumen. Macrophages were, however, commonly located within both the uterine sub-epithelial stroma and the luminal epithelium (Leung *et al.* 2000). Similarly, Cobb & Watson (1995) observed macrophages, T lymphocytes including CD5+ , CD4+ and CD8+ cells and lymphocytic foci in the endometrium of heifers, but in their study, no B-cells were identified.

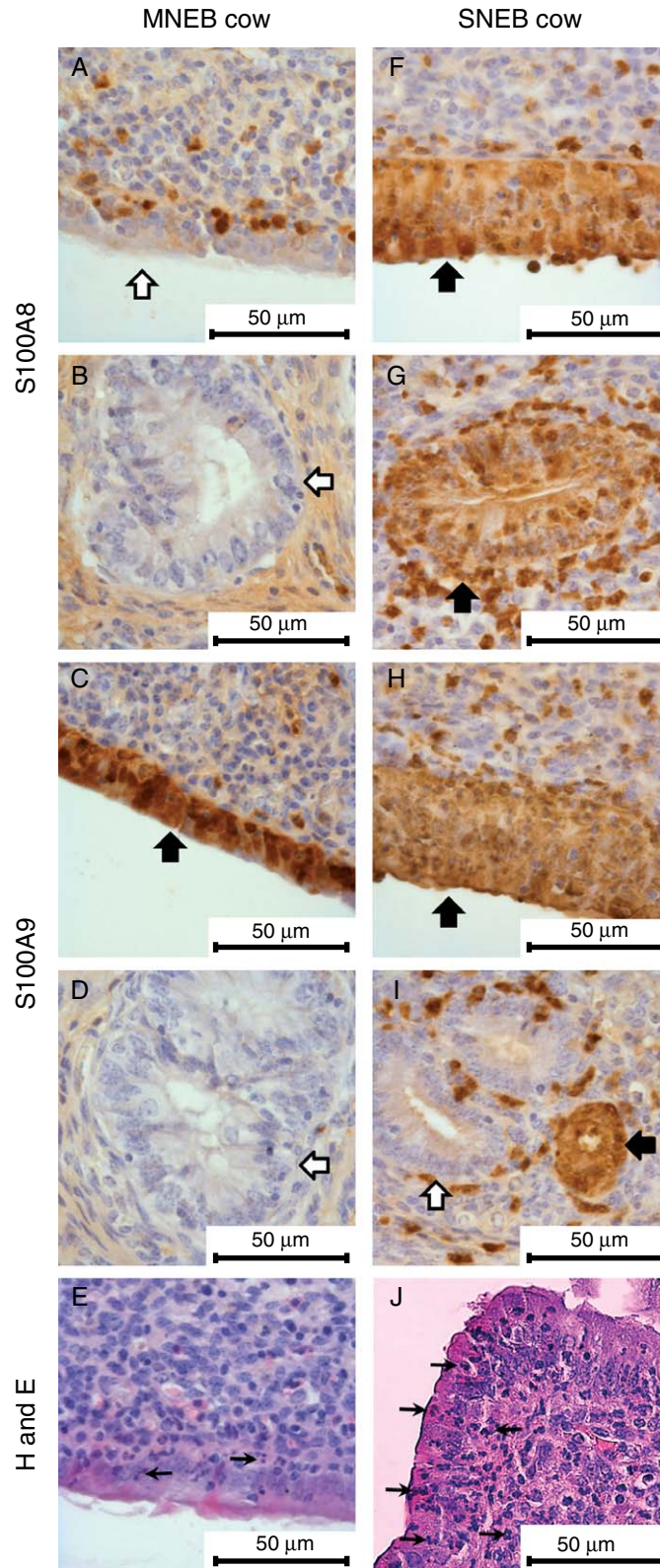


Figure 5 Photomicrographs of *post-partum* bovine endometrium to compare a representative cow in MNEB and one in SNEB. Immunostaining for S100A8 (A, B, F, and G) and S100A9 (C, D, H, and I) in luminal epithelium (A, C, F, and H) and glandular epithelium (B, D, G, and I). Strong positive staining in luminal epithelium and infiltrated leukocytes in both luminal epithelium and stroma is indicated by large closed arrows. Negative to weak staining (open arrows) in luminal and glandular epithelium can also be noted in the MNEB cow. (E and J) H&E-stained sections showing segmented neutrophils in the epithelium and stroma (small arrows).

Table 1 Summary of Pearson correlation coefficients (*r*) between the immunostaining value (H-score) of S100A8 and S100A9 in different endometrial regions from *post-partum* dairy cows^a.

H-score	S100A8				
	IC_LE	IC_GE	IC_ST	C_LE	C_ST
S100A9					
IC_LE	0.912 [†]	0.283	0.521	0.739 *	0.635 *
IC_GE	0.625 *	0.674 *	0.147	0.737 *	0.657 *
IC_ST	0.662 *	0.306	0.890 [†]	0.652 *	0.685 *
C_LE	0.955 [†]	0.607 *	0.540	0.951 [†]	0.896 [†]
C_ST	0.560	0.754 *	0.427	0.686 *	0.726 *

IC, intercaruncular; C, caruncular; LE, luminal epithelium; GE, glandular epithelium; ST, stroma. Correlations highlighted in bold are significant at **P*<0.05 or [†]*P*<0.01 (two-tailed).

^aValues are from unadjusted data and are combined across treatment groups (mild and severe NEB, six cows per group).

PMNs were evident in bovine endometrium undergoing inflammatory processes (Santos *et al.* 2009) but there were fewer in healthy cows (<2% of all samples) (Fischer *et al.* 2010). Lymphocytic foci are commonly present in the bovine uterus after parturition (Bonnett *et al.* 1991) and in animals with endometritis (Hartigan *et al.* 1972). Using immunostaining, we confirmed here the presence of granulocytes, lymphocytes, macrophages, T-cell and NK cells in the bovine *post-partum* endometrium, with clear evidence of cells in the luminal epithelium where they would be best placed to defend against invading microbes.

The endometrium is protected against microorganisms and their toxins not only by physical barriers but also by the production of several effectors such as AMPs and complement via TLRs and other pattern recognition receptors (Sheldon & Bromfield 2011). The work presented here provides the first evidence that the AMPs S100A8 and S100A9 are localised in both epithelial and stromal cells of the endometrium in addition to segmented neutrophils and that protein

levels were significantly higher when cows were experiencing SNEB. Indeed, the majority of immunopositive-stained cells in the *post-partum* endometrium were surface luminal epithelial and stromal cells. Thus, it appears that the endometrium itself functions as the first-line bacterial detector. The significant positive correlations between the H-scores of S100A8 and S100A9 using immunohistochemistry and their mRNA levels as measured by microarray or qPCR confirm the reliability of the measurements made. This was further supported by the significant correlations with *S100A12* but not with *S100A2* mRNA. We have previously reported upregulation of *S100A12* in cultured endometrium challenged with LPS (Swangchan-Uthai *et al.* 2012), indicating a role in immune function, whereas *S100A2* is involved in cell cycle progression and cell migration and is thought to be a biomarker for several cancers (Naz *et al.* 2012). The number of infiltrated segmented neutrophils in endometrium relates directly to the severity of the inflammation (Chapwanya *et al.* 2009). The correlation of immunostaining levels with neutrophil number, particularly in the luminal epithelium, thus firmly links upregulation of S100A8 and S100A9 to an ongoing inflammatory process in response to bacterial contamination after calving.

Defensins and cathelicidins are endogenous mediators primarily involved in cell homeostasis, but they can also activate an immune response as extracellular danger signals when released as a result of cell damage or induced secretion (Yang & Oppenheim 2009). They have thus been termed danger-associated molecular pattern molecules. S100 proteins also appear to function in this way. During endometrial culture, intracellular S100A8 protein levels were high, with little evidence of secretion into the medium (Swangchan-Uthai *et al.* 2012). In *post-partum* cows, however, the ongoing inflammation is associated with

Table 2 Summary of Pearson correlation coefficients (*r*) between the protein levels (H-score) of S100A8 and S100A9 in different endometrial regions from *post-partum* dairy cows with mRNA values measured by qPCR and microarray and with the number of segmented neutrophils in histological samples assessed using H&E staining^a.

H-score (protein)	qPCR <i>S100A8</i>	Microarray results (mRNA)				No. of segmented neutrophils	
		<i>S100A8</i>	<i>S100A9</i>	<i>S100A12</i>	<i>S100A2</i>	Epithelium	Stroma
S100A8							
IC_LE	0.756 *	0.796 *	0.700 *	0.792 *	0.493	0.566	0.747 [†]
IC_GE	0.508	0.596	0.733 *	0.561	0.276	0.748 [†]	0.518
IC_ST	0.034	0.152	0.042	0.129	-0.259	0.180	0.278
C_LE	0.729 *	0.0788 *	0.749 *	0.765 *	0.434	0.651 *	0.680 *
C_ST	0.685 *	0.733 *	0.701 *	0.711 *	0.349	0.684 *	0.622
S100A9							
IC_LE	0.747 *	0.773 *	0.639	0.771 [†]	0.479	0.429	0.697 *
IC_GE	0.576	0.592	0.747 [†]	0.537	0.268	0.604 *	0.615
IC_ST	0.230	0.249	0.221	0.225	-0.225	0.263	0.501
C_LE	0.768 *	0.834 *	0.766 *	0.815 [†]	0.499	0.704 *	0.734 [†]
C_ST	0.506	0.657	0.710 *	0.614	0.328	0.893 [†]	0.577

IC, intercaruncular; C, caruncular; LE, luminal epithelium; GE, glandular epithelium; ST, stroma. Correlations highlighted in bold are significant at **P*<0.05 or [†]*P*<0.01 (two-tailed).

^aValues are from unadjusted data and are combined across treatment groups (mild *n*=6 and severe NEB, *n*=5).

damage to the surface epithelium (Bondurant 1999), which may allow leakage of the S100 family proteins into both uterine secretions and the circulation. S100A8 and S100A9 can form a heterocomplex calprotectin, which is able to inhibit bacterial growth by chelating Zn^{2+} and preventing invasion of organisms through the cell membrane (Steinbakk *et al.* 1990, Nisapakultorn *et al.* 2001). Recently, it has been suggested that the S100A9 subunit was essential to the ability of calprotectin to resist bacterial infection (Champaiboon *et al.* 2009).

TLR4 is the key receptor of the mammalian endometrium, which recognises the LPS component of invading Gram-negative bacteria (Khan *et al.* 2009, Sheldon & Roberts 2010, Chotimanukul & Sirivaidyapong 2011). We previously showed that LPS treatment *in vitro* upregulated S100A8 and S100A9 mRNAs, peaking after 12 h, indicating that this represents a key component of the innate immune response (Swangchan-Uthai *et al.* 2012). There is also strong evidence for an autocrine-positive feedback loop whereby endogenous S100A8 and S100A9 and calprotectin are capable of activating both TLR4 (Vogl *et al.* 2007) and AGER, the receptor for advanced glycation end products (Turovskaya *et al.* 2008), resulting in an amplification of endotoxin-induced upregulation of cytokines, chemokines and other inflammatory mediators (Ehrchen *et al.* 2009).

We previously described measurements of the APP SAA in the same cohort of experimental cows used in this study (Wathes *et al.* 2009). SAA concentrations peaked at day 1–3 *post partum* and then fell by the time of specimen collection around day 14, in agreement with previous studies (Sheldon 2004, Ametaj *et al.* 2009). The average plasma SAA levels in all experimental cows on the day of tissue collection was 92.3 ± 80.0 mg/ml ($n=12$, mean \pm s.d.; range from lower than detection limit to 178 mg/ml). There was no correlation between plasma SAA levels at this time and the parameters recorded in this study, including metabolites, hormones, number of segmented neutrophils and the protein or mRNA levels S100A8 and S100A9 in the endometrial specimens (data not shown). Previous studies have shown that hepatic expression of the SAA1 gene was elevated during the periparturient period in association with increased NEFA levels (Loor *et al.* 2005) and our results from the energy balance model used in this study support the evidence for an acute phase response in early *post-partum* cows with fatty liver. In contrast, endometrial H-scores for S100A8 and S100A9 showed strong positive correlation with the number of segmented neutrophils in endometrial samples. This may indicate that these S100 proteins reflect the severity of endometrial inflammation in *post-partum* cows more closely than other known biomarkers such as the APPs, which are more strongly influenced by hepatic pathology.

In summary, poor energy balance in early *post-partum* dairy cows has a deleterious effect on the degree of

endometrial inflammation. S100A8 and S100A9 were detected locally in the luminal and glandular epithelium and stroma of bovine endometrium as well as in immune cells. It was suggested that these proteins are involved in the activation of cytokines, chemokines and inflammatory mediators through LPS binding to TLR4/MD2 complexes. Upregulation of S100A8 and S100A9 is clearly a key component of the endometrial response to infection in *post-partum* cows. Further studies are warranted to determine possible links in dairy cattle between the extent of this response after calving, the likelihood of developing clinical and subclinical endometritis and subsequent fertility.

Materials and Methods

All chemicals and reagents were purchased from Sigma-Aldrich Company Ltd. or VWR International Ltd. (Poole, Dorset, UK) unless otherwise specified.

Animals and experimental design

All experimental procedures were conducted in accordance with the European Community Directive, 86-609-EC. The experiment was performed at Teagasc, Moorepark Production Research Centre, Ireland, and described previously (Fenwick *et al.* 2008, Wathes *et al.* 2011). In brief, 12 animals from an initial pool of 24 multiparous Holstein-Friesian cows with an average previous lactation yield of 6477 ± 354 kg were used in this study. At 2 weeks before expected calving, the cows were randomly allocated to two interventions designed to produce MNEB or SNEB status. From day 2 or 3 *post-calving*, MNEB cows were fed *ad libitum* grass silage with 8 kg of a 21% crude protein dairy concentrate and milked $\times 1$ per day, while SNEB cows were restricted in dietary intake, i.e. fed with 25 kg grass silage and 4 kg concentrate and milked three times per day. Energy balance status was monitored daily using the French NEL system and presented as UFL per day where 1 UFL is the net energy for lactation equivalent of 1 kg of standard air-dry barley. Measurements of BCS and energy balance status were used to select six cows from each group.

Transrectal ovarian ultrasonography (Aloka SSD-500 7.5 MHz transducer; Aloka Ltd, Tokyo, Japan) was used to monitor ovarian activity daily from day 7 *post partum* to detect the first follicular wave. All cows were killed on days 6–7 after the appearance of the first follicular wave. This design was chosen to collect tissue at a fixed time in relation to the start of ovarian cycles to allow subsequent analysis at the same stage of follicle development. The average *post-partum* interval of MNEB and SNEB groups at killing was 13.6 ± 0.8 and 14.3 ± 0.6 days respectively. Tissue and blood samples were collected at this time.

Blood sampling, metabolite and hormone determinations

Blood samples were obtained from all animals by jugular venipuncture on the day of killing for measurement of

hormones and metabolites. Samples were collected into lithium-heparinized vials and placed on ice before centrifugation at 2000 *g* for 10 min. Plasma was decanted and stored at -20°C for subsequent analysis. Plasma concentrations of metabolites and hormones were measured and reported previously (Fenwick *et al.* 2008). Plasma IGF1 concentrations were determined using human OCTEIA IGF-I kits (IDS, Tyne and Wear, UK), whereas plasma glucose, NEFA, BHB, triglyceride and urea concentrations were measured using commercially available kits (Randox Laboratories Ltd, Crumlin, Co. Antrim, UK) and an ABX Mira autoanalyzer (ABX Mira, Cedex, France). SAA concentrations were measured in serum using the Tridelta Phase range SAA solid-phase ELISA kit according to the manufacturer's guidelines (Tridelta Co., Kildare, Ireland). For hormones, insulin concentrations were determined using the solid-phase RIA Coat-A-Count Insulin kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) and plasma oestradiol concentrations were measured using the Oestradiol MAIA assay kit (BioStat Diagnostic Systems, Cheshire, UK).

Tissue collection

Endometrial specimens were obtained from 12 cows ($n=6$ in each group) enrolled to one of the two treatments designed to produce MNEB and SNEB status. Samples of uterine tissue (caruncular and intercaruncular) from the body, mid-region and tip from both horns were fixed in 4% paraformaldehyde and embedded in paraffin. For assessment of the mRNA levels, samples of intercaruncular endometrial tissue weighing ~ 1 g were dissected from the midportion of the previously gravid horn 1 cm anterior to the bifurcation of the uterus. These were rinsed in RNase-free phosphate buffer, snap-frozen in liquid nitrogen and stored at 80°C .

Histology

Paraffin-embedded samples were sectioned at $10\ \mu\text{m}$ and mounted on glass slides. Endometrial sections of each animal were stained with H&E. The sections were deparaffinised and rehydrated in a graded series of ethanol. The slides were incubated in 10% (v/v) Gill's Haematoxylin No. 3 in dH_2O (Park Scientific Ltd., Northampton, UK) for 4 min and washed with running tap water for 5 min. They were then placed in dH_2O before incubating in 1% (w/v) eosin (Sigma) in dH_2O for 1 min and washed in dH_2O (2×2 min). The stained sections were dehydrated and mounted with VectaMount permanent Mounting Medium (Vector Laboratories, Peterborough, UK) before applying coverslips.

The H&E-stained specimens were used to assess the degree of uterine inflammation as described by Bonnett *et al.* (1991). Inflammatory cells were subdivided into segmented (neutrophils) or mononuclear cells (macrophages and lymphocytes). The number of segmented and mononuclear cells was the mean of three sections per horn (gravid and non-gravid), taken in each case from the uterine tip and a caruncular region and an intercaruncular region from the mid-region under a Nikon 187907 light microscope ($\times 400$ magnification). Those in epithelium were expressed as number per graticule length and those in stroma as the average number of cells per $10\ \mu\text{m}^2$. The

number of lymphocytic foci was counted in stroma under a light microscope ($\times 100$ magnification) and was expressed as the number per section. Capillary density in the stroma (stratum compactum) was measured as the number of cross sections in $20\ \mu\text{m}^2$ averaged over four sites per section.

Immunohistochemistry

Samples from previously gravid uterine horns were subjected to optimised immunoperoxidase staining for proteins S100A8 and S100A9 and leukocyte markers. In addition, liver and ovarian tissues were used as positive and negative control tissues respectively for S100A8 and S100A9. The paraffin-embedded samples were cut into $6\ \mu\text{m}$ -thick sections, applied onto HCl-treated poly-L-lysine-coated slides and left to dry in an incubator at 37°C for 24 h before use.

Immunohistochemical localisation of proteins S100A8 and S100A9 was performed using an immunoperoxidase staining procedure, Vectastain Elite ABC Kit (Vector Laboratories), in accordance with guidelines supplied by the manufacturer. Tissue sections were deparaffinised in Histo-Clear tissue clearing agent (Fisher Scientific, Loughborough, UK) and rehydrated in a graded series of ethanol. The sections were immersed in 0.3% hydrogen peroxide in methanol for 60 min to inactivate endogenous peroxidase, washed in PBS pH 7.5 twice for 5 min each and then subjected to microwave pre-treatment for 7 min twice at 700 W in 0.01 M citrate buffer solution (pH 6.0) for antigen retrieval and left to stand at room temperature for a further 30 min. After being washed in PBS (2×5 min), the sections were incubated with blocking solution (1% (v/v) horse serum and 20% (v/v) avidin solution (Vector Laboratories) in PBS) for 30 min to suppress non-specific binding. The sections were then incubated in a humidified chamber with primary antibodies or control antibody overnight at 4°C . The primary antibodies used were mouse mAbs against human S100A8 (BMA Biomedicals, Augst, Switzerland) and S100A9 (BMA Biomedicals). Both mAbs against S100A8 and S100A9 are specific for the target protein and cross-reactivity between S100A8 and S100A9 was minimal (according to the manufacturer) as measured in a direct ELISA with recombinant human proteins. Considerable cross-reactivity was observed for the human-derived antibodies on bovine tissue (manufacturer's datasheet). Each antibody was thus specific for the target S100 protein. These primary antibodies were diluted at a concentration of $0.2\ \mu\text{g}/\text{ml}$ in PBS and 20% (v/v) biotin solution (Avidin/Biotin blocking kit; Vector Laboratories). The negative control sections were treated in the same manner with $0.2\ \mu\text{g}/\text{ml}$ mouse IgG (Vector Laboratories) diluted with PBS and biotin solution. After being washed in PBS (3×5 min), the sections were incubated with the secondary antibody ($40\ \mu\text{g}/\text{ml}$ biotinylated anti-horse IgG in PBS (Vector Laboratories) and 1.6% (v/v) horse serum) for 1 h at room temperature, washed in PBS (3×5 min), and treated in the avidin-biotin complex solution for 30 min at room temperature. The sections were then washed in PBS (3×5 min) and developed with 3% (v/v) 3,3'-diaminobenzidine (ImmPACT DAB Chromogen concentrate) in ImmPACT diluent (ImmPACT DAB Peroxidase substrate; Vector Laboratories) for 1 min under dark conditions. Positive staining was brown colour and no staining was observed for negative

controls. The slides were then placed in dH₂O for 5 min twice before being subjected to perform contrast staining using 10% (v/v) Gill's Haematoxylin No. 3 in dH₂O (Park Scientific Ltd.) for 30 s and washed with running tap water for 5 min. The stained sections were dehydrated in a graded series of ethanol and mounted with VectaMount permanent Mounting Medium (Vector Laboratories) before applying the coverslips.

The presence of inflammatory cells was confirmed using immunostaining methods with the following specific mAbs: CD172A mAb DH59B (macrophage and granulocyte marker; VMRD, Inc., Pullman, WA, USA, used at 1:100 dilution); CD14 mAb MCA1568F (macrophage marker, AbD Serotec, Kidlington, UK, used at 1:50); CD2 (mAb CC42, T-cell and NK cell marker, used at 1:5) and CD8 (mAb CC31, cytotoxic T-cell marker, used at 1:10). The CD2 and CD8 antibodies were kind gifts of Prof. D Werling, Royal Veterinary College, London. The procedures were similar to those described earlier. On day 2, slides were washed with PBS (2 × 5 min) and incubated for 1 h with dilute secondary antibody using an ABC kit (Vectastain Vector Laboratories) according to the manufacturer's instructions.

Quantification of immunohistochemistry

Five ROIs selected for analysis were as follows: the luminal epithelium, glandular epithelium and stroma of the intercaruncular endometrium and the luminal epithelium and stroma of the caruncular endometrium, both taken from the previously gravid horn. Endometrial sections were photographed using an Olympus BX-60 microscope by one operator unaware of the treatment by use of a random number system. Images from the entire stained sections were captured in full colour at ×400 magnification and saved as high-resolution digital images (TIFFs). A total of ten images per slide were obtained from each cow, where each slide contained two to three sections. The intensity of staining was scored by a user-defined digital quantitative image analysis system (Volocity 5.5; PerkinElmer, Inc., Waltham, MA, USA) and classified on a scale of 0–3, where 0, negative staining; 1, weak staining; 2, moderate staining and 3, strong staining. For statistical analysis, immunostaining of S100A8 and S100A9 was evaluated using a histology score (H-score). The H-score was calculated from the intensity and area proportion scores using the following equation: $H\text{-score} = (1 \times \% \text{ area of score } 1) + (2 \times \% \text{ area of score } 2) + (3 \times \% \text{ area of score } 3)$, giving a possible range of 0–300 (Pierceall *et al.* 2011). The same scoring method was used separately on the different ROIs of either caruncular or intercaruncular endometrium from each animal for both proteins S100A8 and S100A9.

RNA preparation, microarray analysis and real-time qPCR

Total RNA was extracted from 200 to 300 mg frozen endometrial tissue from each cow using TRI Reagent (Molecular Research Centre, Cincinnati, OH, USA). RNA concentration and purity of each sample was evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), where all samples had an acceptable 260:280 ratio of absorbance between 1.8 and 2.1. RNA integrity was

confirmed for all samples using automated capillary gel electrophoresis on a Bioanalyzer 2100 with RNA 6000 Nano Labchips according to the manufacturers' instructions (Agilent, Waldbronn, Germany).

Microarray analysis was performed previously using 24K AffymetrixGeneChip Bovine Genome Arrays (Wathes *et al.* 2009). Only the data for S100A2, S100A8, S100A9 and S100A12 were used in this study. The mRNA concentration of S100A8 was also determined by a real-time qPCR procedure with a single-plex SYBR Green I assay and reported previously (Wathes *et al.* 2009). RPL19, GAPDH and RNAT8S5 (RN18S1) were tested as reference genes. Analysis of relative quantification from the ratios of the mRNA concentrations of the genes of interest to the reference genes was carried out in parallel with the absolute quantification. However, levels of all three reference genes were not significantly different between SNEB and MNEB groups ($P \geq 0.05$). The absolute value of mRNA (fg/μg reverse-transcribed RNA) was therefore used in this study.

Statistical analysis

Statistical analysis of the data was carried out using SPSS, V18.0. A linear mixed effect model was used for analysis of the effect of NEB status and ROIs on the level of immunostaining H-scores for S100A8 and S100A9. This model included NEB status (SNEB and MNEB), ROIs (intercaruncular luminal epithelium, glandular epithelium and stroma, and caruncular luminal epithelium and stroma) as the fixed effects and animal as the random effect. *Post hoc* comparisons were performed using the Bonferroni test. Data were tested for homogeneity of variance using a Levene test and log transformation was performed if necessary.

Differences in H-score, histological measurement and the number of epithelial and stromal leukocytes in the endometrial sections between MNEB and SNEB groups were analysed using an independent samples *t*-test. Levene test was used to account for variance, and if homogeneity was not achieved, an unequal variance *t*-test was used.

Relationships between the protein measured by immunohistochemistry staining, plasma metabolite and hormone parameter, mRNA values from microarray data and real-time quantitative PCR, and the number of leukocytes in the histology samples were determined from values pooled across treatment groups. The spread of data was tested for normality using the Shapiro–Wilk test and log transformations were performed if appropriate before performing Pearson correlations. The false discovery rate was used to adjust the level of significance to account for multiple comparisons (Benjamini & Hochberg 1995). Results from all analyses are presented as sample mean ± S.E.M. Statistical significance was considered at a *P* value of 0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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