



## Influence of subclinical endometritis on the reproductive performance of dairy cows

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### Abstract

The aim of this study was to evaluate the influence of subclinical endometritis (SE) on the reproductive performance of dairy cows. Ninety-four dairy cows of parity 1 to 8, distributed in 25 herds, were examined once between 30 and 45 days in milk using transrectal palpation, vaginoscopy and ultrasonography. A cytological sample of the endometrium was taken only from cows with an apparent healthy uterus (n=65). Serum glucose, total cholesterol, triglycerides, non-esterified fatty acids,  $\beta$ -hydroxybutyrate, total proteins, albumin, urea and hepatic enzymes were analyzed. Reproductive indexes were recorded during the next 11 months. Endometrial cytology was considered indicative of SE if percentage of polymorphonuclear neutrophils was superior to 5% of all cells present in the smear, except erythrocytes. Results indicated that 14.9% of the cows sampled for uterine cytology had SE, and that healthy cows become pregnant significantly before than those with SE (hazard ratio=2.35; 95% confidence interval: 1.05-5.3). From all the metabolic and productive variables analyzed, only triglycerides affected negatively to reproduction; serum albumin concentration, body condition score and milk production had positive effects on the reproductive performance. In conclusion, our results indicate that SE has a negative impact on reproductive performance and uterine cytology is necessary to diagnose it since almost 15% of the affected animals were not detected by other diagnosis methods.

**Additional key words:** subclinical endometritis; dairy cattle; ultrasound; endometrial cytology.

**Abbreviations used:** ALAT (alanine transaminase); ASAT (aspartate aminotransferase); BCS (body condition score); BHB (beta-hydroxy butyrate); CI (confidence interval); HR (hazard ratio); NEB (negative energy balance); NEFA (non-esterified fatty acid); PMN (polymorphonuclear neutrophil); S/C (services per conception); TAG (triglycerides); US (transrectal ultrasonography).

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The postpartum period is considered of utmost importance in the reproductive life of the cow because of its influence upon her future fertility (Oltenacu *et al.*, 1983). Uterine contamination at parturition or in the following days is considered normal, with 80-100% of animals having bacteria in the uterine lumen in the first 2 weeks postpartum (Sheldon *et al.*, 2006). The development of clinical disease is dependent on the balance between host immunity and bacteria pathogenicity (Grohn & Rajala-Schultz, 2000). The primary defense mechanism of the bovine uterus is

phagocytosis of contaminating organisms by neutrophils. The presence of neutrophils in the uterine lumen is an excellent indicator of an active inflammatory process (Wade & Lewis, 1996). This is especially important in subclinical endometritis (SE) in which physical examination of the reproductive tract may not reveal evidence of inflammation (Mateus *et al.*, 2002). SE is characterized by inflammation of the endometrium that results in a significant impairment of the reproductive performance without signs of clinical endometritis (Sheldon *et al.*, 2006). Among

the commonly used diagnostic tools, rectal palpation is probably the most frequently used for diagnosis of uterine infections, but it may be the most insensitive and non-specific method (Gilbert & Schwark, 1992). Vaginoscopy is a straightforward procedure that permits the evaluation of the characteristics of fluids in the anterior vagina and external cervical os (Bretzlaff, 1987). Transrectal ultrasonography (US) has also been used to detect intrauterine fluid accumulation (Kasimanickam *et al.*, 2004; 2005) or cervical diameter (LeBlanc *et al.*, 2002) and in spite that it offers the advantage of immediate diagnosis, its utility in SE can be limited. The most precise procedure for diagnosing endometritis was demonstrated to be cytobrush cytology (Barlund *et al.*, 2008), which can help to solve the negative impact of this uterine affection on the reproductive performance of dairy cattle. However, cytology is not frequently used in the daily practice because the results are not immediate, and it is more time-consuming than other diagnostic methods.

Milk yield increases at a faster rate than energy intake in the first 4 to 6 weeks after parturition, consequently high producing cows will experience some degree of negative energy balance (NEB) and a negative balance of other nutrients during early postpartum period. NEB has been positively correlated with serum concentrations of triglycerids (TAG) and  $\beta$ -hydroxybutyric acid (BHB), and negatively associated with serum concentrations of glucose and cholesterol in periparturient cows (Lean *et al.*, 1992). Elevated liver TAG are associated with a longer interval to first ovulation and with reduced fertility (Butler & Smith, 1989). It is well known that progesterone concentrations (Villa-Godoy *et al.*, 1988), estrus behavior (Spicer *et al.*, 1990) and days to first ovulation (Ducker *et al.*, 1985) are all affected by the degree of NEB in early lactation.

The objective of our field trial was to determine the prevalence of SE, diagnosed by uterine cytology, in a group of dairy cows diagnosed as having a healthy uterus by rectal palpation, vaginoscopy or US, and its impact on the postpartum fertility. Potential confounders in reproductive performance such as metabolic disorders or production level were considered in the analysis.

For this study, Holstein cows (1-8 parities) from 25 farms located in the NW of Spain were used. The average farm size was 35 animals, ranging from 15 to 90. Average annual milk production (305-day) was 8,610 kg. In 15 farms animals were housed in tied stall barns and in the other 10 they were in free stall with cubicles, and all used mixer wagon to prepare rations. Herds were maintained on monthly or biweekly reproductive control programs.

All animals calving between October 2008 and February 2009 were evaluated for reproductive status between 30-45 days in milk (DIM) and, if presumed to be in the luteal phase of the estrous cycle or anestrous, were included in the study and a blood sample was taken. Cows in estrus were not used. A total of 94 cows were finally selected and examined by rectal palpation, vaginoscopy and US. Cows with any detectable sign of endometritis (abnormal discharge of uterine fluid, abnormal fluid into the uterus or pathologic enlargement of the uterus) diagnosed by any of the three methods, were classified as having clinical endometritis and excluded from the study (n=29). Cows with no detectable signs of endometritis were sampled for uterine cytology (n=65). Cows in their first month postpartum were not used because uterine involution might not have finished (Gautam *et al.*, 2009) and then, a high percentage of polymorphonuclear neutrophil (PMN) in the uterine cytology would be a normal finding.

Uterine cytology was obtained using a cytobrush (Quicklock 2000, Minitube Iberica, Barcelona, Spain) twisted to the plunger of an insemination catheter (modified from Kasimanickam *et al.*, 2004). The cytobrush was rolled onto a clean slide and allowed to air-dry. Slides were stained by Diff Quick (Quick Panoptic®, Tarragona, Spain). All the slides were examined by the same person using an optic microscope (CHT, Olympus, Barcelona) at 400X magnification. A minimum of 150 cells (excluding erythrocytes) were counted on each slide to provide a quantitative assessment of endometrial inflammation. Samples with  $\geq 5\%$  neutrophils were categorized as SE positive (Gilbert *et al.*, 2005). Results of cytology were not reported to farmers to prevent individual actions on the positive cows and only investigators knew such information.

After genital examination, a blood sample was taken by venipuncture of the coccygeal vein. Serum samples were separated by centrifugation and stored at  $-20^{\circ}\text{C}$  until analysis. Analyses were done with a digital photometer (Selecta MD200, Barcelona), except total proteins that were analyzed with a portable refractometer (SELECTA, Barcelona). Glucose, total cholesterol, TAG and albumin concentrations were determined by a colorimetric endpoint method by using Biosystems® reagents (Biosystems, Barcelona); hepatic enzymes (Alanine transaminase [ALAT], Aspartate aminotransferase [ASAT]) were analyzed by the IFCC (International Federation of Clinical Chemistry) method (Tietz, 1991) using Biosystems® reagents; urea was assayed by a colorimetric enzymatic method using Spinreact® reagents (Spinreact, Sant Esteve de Bas, Spain) and non-esterified fatty acids (NEFA) and BHB were analyzed by kinetic enzymatic kits (Randox Laboratoires, Antrim, UK).

Animal specific data (body condition score [BCS], parity, parturition date), periparturient disorders (assisted calving, retained fetal membranes, abortion, hypocalcaemia, ketosis, mastitis, etc.) were recorded. Starting from the day of the reproductive exam, insemination dates, pregnancy diagnosis dates and milk production were obtained from the herd reproductive management software, during 11 months following samples collection.

A Kaplan-Meier survival analysis was used to compare calving to conception and to first insemination intervals and services per conception (S/C), both in animals with or without SE. Survival analysis was used to examine the number of S/C considering each service as a "time period" (Gilbert *et al.*, 2005).

Parity, production in 305-day, body condition, biochemical profile (glucose, NEFA, BHB, total cholesterol, TAG, total proteins, albumin, urea, ASAT, ALAT) and cytology results were included in a Cox's proportional hazards regression model for calving to conception and calving to first insemination intervals and for S/C. All the independent variables were confirmed to comply the proportional hazards assumption. Models were built by reverse stepwise elimination using likelihood-ratio test statistics, with threshold *p* values <0.1 for inclusion and >0.15 for exclusion.

The Kaplan-Meier estimated median times from calving to conception, calving to first insemination and S/C are shown in Table 1. Cows with negative endometrial cytology became pregnant earlier (HR=2.35; 95% CI: 1.05-5.29) than those with SE (Fig. 1).

Only TAG and albumin had significant effects on the interval calving to conception according to the Cox model. Increased levels of albumin or reduced levels of TAG were associated to shorter calving to conception intervals (Table 2). Moreover, increase of milk production was associated with a shorter calving to conception interval. No other metabolic variables had relationship with the interval calving to conception.

Calving to first insemination interval and number of S/C were not significantly different in healthy animals *vs.* those with SE. Only TAG levels had an effect on the number of S/C and BCS had an effect on calving to first insemination interval (Table 2).

In the present study, the prevalence of SE (14.9%) found between 30 and 45 DIM was lower than that reported by other studies [34%, Kasimanickmam *et al.* (2004); 53%, Gilbert *et al.* (2005)] and higher than that reported by Lopdell *et al.* in 2011 (7%). Such discrepancies can be due to the different DIM periods chosen for the diagnosis, different cut offs for PMN%, or to different management systems of the farms studied. One of the reasons for the low SE prevalence found in this study was the criterion used for the diagnosis, as a cow was considered with SE when the condition was diagnosed exclusively by endometrial cytology. In addition, any amount of echogenic fluid in the uterus was considered a sign of clinical endometritis (Barlund *et al.*, 2008). Therefore, we classified as clinical endometritis some cases that other researches would have considered to be subclinical (Kasimanickman *et al.*, 2004). The PMN cut off used for establishing the limit

**Table 1.** Results [median (confidence interval)] of Kaplan-Meier analyses for calving to conception and calving to first insemination intervals and services per conception

	n	Calving to conception interval	Calving to first insemination interval	Services per conception
Subclinical endometritis	14	154 (82.50-225.50)	77 (64.17-89.83)	3 (1.90-4.11)
No endometritis	51	119 (88.14-149.86)	68 (61.91-74.10)	2 (1.10-2.90)
<i>p</i> value	65	0.09	0.11	0.19

**Table 2.** Factors affecting reproductive variables obtained with Cox regression analyses

Dependent variable	Variables in the equation <sup>1</sup>	<i>p</i> -value	HR <sup>2</sup>	95% CI of HR <sup>2</sup>
Calving to conception interval	Production*100 (305-d)	0.05	0.98	0.96-1.00
	TAG (mg/dL)	0.01	0.92	0.87-0.98
	Albumin (g/L)	0.05	1.05	1.00-1.10
	PMN < 5%	0.04	2.35	1.05-5.29
Calving to first insemination interval	BCS	0.008	2.62	1.29-5.34
Services per conception	TAG (mg/dL)	0.02	0.94	0.88-0.99

<sup>1</sup> TAG, triglycerides; PMN, polymorphonuclear neutrophil; BCS, body condition score; <sup>2</sup> HR, hazard ratio; CI, confidence interval.

between healthy animals and those with SE was slightly inferior than that used by other authors (Kasimanickam *et al.*, 2004; Sheldon *et al.*, 2008), thus, this factor was not likely to be responsible for the lower prevalence found in this trial. Neither have we attributed the difference to the cytology technique, since cytobrush is believed to be more sensitive than uterine washing for detecting SE (Kasimanickam *et al.*, 2005).

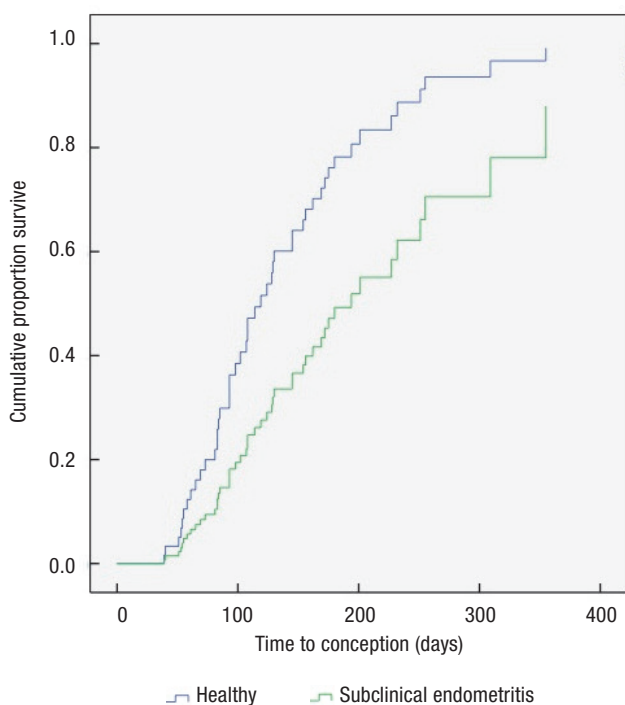
Studies in dairy cows have shown an increase in days open and a diminished likelihood of pregnancy before 300 d postpartum for cows cytologically diagnosed with endometritis after 40 or 50 d postpartum (Gilbert *et al.*, 2005). Likewise, the present results showed an effect of SE on the interval from calving to conception: 80% of healthy cows were pregnant within 200 days postpartum whereas only 60% of cows with SE did (Fig. 1).

The role of PMN in inflammatory processes is the rapid inactivation and elimination of foreign and altered autologous structures. In addition, they exert various regulatory functions such as release of cytokines, priming, activation or inhibition of cells, or influencing the expression of surface molecules (Baggiolini *et al.*, 1993). Certain cytokines can decrease luteinizing hormone release (McCann *et al.*, 2000) causing a delay in the onset of postpartum cyclic activity and increasing the interval calving to first insemination. In the present study, the interval calving to first insemination was not

significantly different for the two groups of animals (68 d for healthy cows *vs* 77 d for cows with SE). Although the two individual variables (longer interval calving first service and higher number of S/C) had no significant effects, both together contributed to the significantly longer interval calving to pregnancy found in this trial in cows with SE.

Reproductive problems may be associated to imbalances of the animals' metabolic status. With the purpose of isolating the effect of SE, several metabolic variables were included in the Cox model so to assess their potential influence on reproduction. The need to analyze metabolic parameters arose because several farms did not have good records of metabolic diseases occurrence, or not always were diagnosed, as for example subclinical ketosis. Only TAG, albumin, BCS and milk production had significant effects on reproduction. We found that TAG affected negatively to the interval calving to conception, and BCS affected negatively to the interval calving to first insemination. TAG accumulate in liver after body fat mobilization and they are considered good indicators of NEB (Rukkamsuk *et al.*, 1999). Other authors related high levels of TAG in liver (Butler & Smith, 1989) and NEB (Beam & Butler, 1999) with a long interval between calving and first ovulation, and with low fertility. Levels of BHB (Kessel *et al.*, 2008), NEFA (Bronicki *et al.*, 1996) and urea (Moreira da Silva *et al.*, 1997) have been associated to adverse reproductive parameters, but such effects were not seen in this study. Nevertheless, the small number of cows diagnosed with SE in this study may have masked the influence of these variables on reproduction. On the other hand, metabolic parameters were analyzed during the second month postpartum, and perhaps this is not the best moment to determine their effect on reproduction, as the NEB is known to be more severe during the first 30 d (Cavestany *et al.*, 2005). By contrast, albumin had a positive effect on reproductive efficiency, probably linked to the correct function of liver, since albumin was found to be good indicator of a healthy liver (Lubojacká *et al.*, 2005).

Definitive conclusions could not be drawn from this study as the number of cows diagnosed with subclinical endometritis between 30 and 45 days in milk was low (14) and therefore, the real impact of the metabolic status of the cows on reproduction could not be accurately determined. Nevertheless, a negative effect of subclinical endometritis on reproductive performance was observed. Endometrial cytology can be used as a rapid and easy method to diagnose subclinical endometritis and should be recommended to practitioners as a number of animals suffering subclinical endometritis cannot be diagnosed by rectal exploration, transrectal ultrasonography or vaginoscopy.



**Figure 1.** Calving to conception interval based on the presence or absence of subclinical endometritis.

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