



Universitat de Lleida

From parturition to pregnancy. A clinical perspective in the dairy COW.

Irene López Helguera

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**From parturition to pregnancy.
A clinical perspective in the dairy cow.**

DISSERTATION

To obtain the degree of Doctor at the University of Lleida

by

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Cover pictures:

First row, from left to right: 7.5 MHz- Ultrasonography of a cross section of cervical diameter, ovarian structures (two corpus luteum and a follicle) and cross section of a uterine horn.

Second row, from left to right: 7.5 MHz- Ultrasonography of a cross section of a uterine horn with echogenic intrauterine fluid, increased endometrial thickness and echogenic intrauterine fluid, respectively.

I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

Marie Curie

INDEX

Summary	– English	7
	– Català	9
	– Castellano	11
General Introduction	13
Main objectives	27
Studies		
Chapter 1	31
The influence of genital tract status in postpartum period on the subsequent reproductive performance in high producing dairy cows		
Chapter 2	53
Therapy hormonal treatment for abnormal uterine involution on high producing dairy cows.		
Chapter 3	73
Is intrauterine therapy of abnormal uterine involution useful in high producing dairy cows?		
Chapter 4	93
Reproductive performance of anoestrous high-producing dairy cows improved by adding equine chorionic gonadotrophin to a progesterone-based oestrus synchronization protocol.		
General Discussion	115
Conclusions	129
Epilogue	133
Appendix	– Acknowledgements	145
	– Curriculum vitae	147

SUMMARY

Nutritional and genetic improvements in last decades have not prevented the decrease in reproductive performance of dairy cows. For that reason, great effort has been focused on understanding pathological processes during early postpartum. Therefore, the aims of this thesis were to implement a diagnostic and therapeutic protocol on Days 15-21 postpartum that detect cows suffering endometritis and to improve subsequent reproductive performance in high producing dairy cows. Moreover, since there is still no one single gold protocol to synchronize lactating dairy cows, progesterone based synchronization protocol plus equine chorionic gonadotrophin (eCG) was tested. The research included in this thesis was divided in four studies that are summarized below.

In the first study, cows suffering postpartum endometritis on Days 15-21 were characterized by ultrasonography, endometrial cytology and blood tests. Measures of cervical diameter, endometrial thickness and echogenicity of intrauterine fluid (IUF) by ultrasonography on Days 15 to 21 postpartum were a good tool to predict subsequent reproductive performance in clinically normal dairy cows.

In the second one, the effect of Prostaglandin F_{2α} on Day 15-21 postpartum on subsequent conception rate was evaluated. No effect of treatment with prostaglandin was observed on subsequent reproductive performance even in cyclic cows. Early resumption of ovarian cyclicity enhanced postpartum recovery and improved subsequent conception rate.

In the third study, the effect of a single intrauterine infusion of penicillin or large-volume lavage with normal saline solution on cows suffering endometritis on Days 15-21 postpartum were assessed. No effect was observed of treatment on the subsequent conception rate or return to cyclicity. Factors such as calving season and parity were intimately related to reproductive performance.

In the last work, a progesterone based synchronization protocol plus eCG was tested. The placement of a progesterone intravaginal device followed by eCG treatment given on the day of device removal was beneficial in anoestrous cows even during the postpartum period. Irrespective of the treatment, cows undergoing double ovulation were more fertile than the remaining cows.

RESUM

Les millores en genètica i nutrició de les últimes dècades, no han impedit que es produís una disminució de la funció reproductiva en les vaques lleteres d'alta producció. Per aquest motiu, entendre els processos fisiopatològics durant el postpart ha estat el principal motiu d'atenció de les granges lleteres. Per tant, l'objectiu d'aquesta tesi va ser implementar un protocol diagnòstic i terapèutic en vaques lleteres a dies 15-21 postpart per detectar animals que patien endometritis i millorar la seva eficàcia reproductiva posterior. A més, degut a que no existeix un protocol estandaritzat per sincronitzar vaques lleteres, es va implementar un protocol basat en progesterona intravaginal més gonadotrofina coriònica equina (eCG). La recerca inclosa en aquesta tesi està dividida en quatre estudis:

En el primer, es van caracteritzar animals que patien endometritis a dies 15-21 postpart utilitzant ecografia transrectal, citologia uterina i anàlisi de cèl·lules sanguínies. Les mesures de tamany cervical, gruix endometrial i ecogenicitat del fluid intrauterí caracteritzades per ecografia van resultar ser una eina útil per predir la funció reproductiva posterior en vaques clínicament normals.

En el segon, es va estudiar l'efecte de la prostaglandina $F2_{\alpha}$ a dies 15-21 postpart sobre la fertilitat posterior. No es va observar efecte del tractament ni tan sols en les vaques que ja eren cícliques. El precoç retorn a la ciclicitat millorava la recuperació postpart i millorava la posterior fertilitat.

En el tercer estudi, es va establir l'efecte d'un rentat intrauterí amb penicilina o sèrum salí fisiològic en vaques que patien endometritis a dies 15-21 postpart. No es va observar efecte del tractament ni a la posterior fertilitat ni al retorn a la ciclicitat. Factors com la estació de part o la lactació resultaren intimament relacionades amb la funció reproductiva.

Per últim, es va establir un protocol basat en progesterona intravaginal i eCG el dia de la retirada. Aquest protocol va ser beneficiós tant en vaques que patien llarg anestre com en animals amb anestre postpart. Independentment del tractament, les vaques amb doble ovulació van ser més fèrtils que la resta.

RESUMEN

La mejora genética y nutricional de las últimas décadas no ha logrado frenar la disminución del rendimiento reproductivo en las vacas de leche. Los procesos fisiopatológicos que ocurren durante el postparto son esenciales para la fertilidad posterior, siendo actualmente un importante punto de estudio. Por lo tanto, el objetivo de esta tesis fue establecer un diagnóstico y un protocolo terapéutico en los días 15-21 del postparto para detectar las vacas que sufren endometritis y mejorar la eficacia reproductiva en vacas lecheras de alta producción. Por otra parte, debido a que todavía no hay un único protocolo estandar para sincronizar la vacas de leche en lactación, se estableció un protocolo basado en la sincronización con progesterona aplicando gonadotropina coriónica equina (eCG). La investigación incluida en esta tesis se divide en cuatro estudios que se resumen a continuación.

En el primer estudio, se analizaron vacas con endometritis postparto entre los días 15-21 por ecografía, citologías y análisis de sangre. Las medidas del diámetro cervical, el grosor endometrial y el fluido intrauterino ecogénico (IUF) por ecografía entre los días 15 al 21 de postparto, resultaron ser una buena herramienta para predecir la posterior eficacia reproductiva en vacas de leche clínicamente sanas.

En el segundo estudio, se evaluaron los efectos de la prostaglandina $F2_{\alpha}$ en los días 15-21 postparto sobre la posterior tasa de concepción. No se observó efecto del tratamiento ni siquiera en las vacas cíclicas. El pronto retorno de la ciclicidad ovárica después del parto se relacionó positivamente con la recuperación postparto y la tasa de concepción posterior.

En el tercer estudio, se evaluaron los efectos de un único lavado intrauterino con penicilina o suero fisiológico en vacas con endometritis a días 15-21 postparto. No se observó efecto del tratamiento ni en la posterior fertilidad ni en el retorno a la ciclicidad. Factores como la estación del parto o el número de lactación estuvieron íntimamente relacionados con la función reproductiva .

En el último trabajo, se estableció un protocolo basado en progesterona intravaginal y eCG el día de la retirada. Este protocolo resultó beneficioso, tanto en vacas con anestro largo como en animales con anestro postparto. Independientemente del tratamiento, la vacas con ovulación doble fueron más fértiles que el resto.

INTRODUCTION

INTRODUCTION

Management and genetics of dairy cows have been improving along years, leading to a continuous increase in milk production (Dobson *et al.* 2007; Yániz *et al.* 2008). However, since 1980s subfertility has become an emerging disease (Lucy 2001; López-Gatius 2003), reducing farm productivity. For instance, economic consequences of non-optimal fertility are estimated at 34-231 Euros per cow/year (Inchaisri *et al.* 2010). It is well known, that high milk production correlates negatively with fertility (Pryce *et al.* 1998). However, this relationship is difficult to demonstrate because of the interference of culling and the environment (Montaldo *et al.* 2010). A high milk yield will only provoke a higher risk of infertility under suboptimal conditions (García-Ispuerto *et al.* 2007), such as postpartum period (Walsh *et al.* 2011). For that reason, great effort has been focused on understanding physiological changes and pathological processes during postpartum and voluntary waiting period. Nevertheless, too much questions are not clearly understood yet.

The fetal hypothalamo-pituitary-adrenal axis stimulates the production of corticoids following nine months of gestation. Then, a massive increase in cortisol and decrease in progesterone triggers the onset of parturition (Senger 1997). These endocrine patterns determine the number and function of immune cells in utero that potentially affects uterine defense mechanisms postpartum (Singh *et al.* 2008). The potentially immunosuppressed animal has to suffer for several weeks after parturition a cycle of bacterial contamination, clearance, and recontamination (Griffin *et al.* 1984; Sheldon and Dobson 2004). Whether these animals in negative energy balance due to their reduced food intake and increased milk production, are not able to resolve this natural contamination, their subsequent reproductive performance will be substantially impaired (Hammon *et al.* 2006; Goff 2008).

Before a cow is likely to conceive again, it has to reach two main objectives: undergo uterine involution and return to ovarian cyclicity (Morrow *et al.* 1969, Sheldon *et al.* 2008). Placenta has to be expelled before 12 hours, although loquial discharge could be present during the first two weeks after calving and endometrial regeneration is not completed until 6-8 weeks postpartum (Sheldon 2004). However, during pregnancy,

the hypothalamic pituitary axis is suppressed by steroidal hormones, reducing the secretion of luteinizing hormone (LH). After parturition, steroidal hormones return to basal levels and ovarian dynamics should be reestablished (Crowe 2008). Both phenomena, uterine involution and return to cyclicity, greatly interact so that early postpartum ovarian activity could help to bacterial uterine elimination because of the increase of estrogens (Hussain 1989; Lewis 2004). Despite of the positive effect of estrus, an early ovulation may provoke an untimely cervical closure leading to a pyometra (Olson *et al.* 1984). The persistence of bacterial uterine contamination could not only damage endometrial tissue and compromise its ability to produce or transport PGF_{2α}, but also suppresses GnRH and LH secretion (Olson *et al.* 1984; Sheldon and Dobson 2004).

Diagnosing and treating postpartum diseases is essential for the subsequent reproductive performance of high producing dairy cows. Clinical diseases, such as puerperal metritis are easily diagnosed by observing clinical signs like fetid red-brown watery uterine discharge and pyrexia within 10 days after parturition (Sheldon *et al.* 2006). However, on the following days, diagnosis can be much more difficult. Endometritis, inflammation of the endometrium without affecting the remaining uterine layers, is usually diagnosed by vaginoscope or observing vaginal discharge after day 21 postpartum (LeBlanc *et al.* 2002; Sheldon *et al.* 2006). Thus, this uterine pathology can be underestimated when the cervix closes rapidly after parturition (Kasimanickam *et al.* 2004). Consequently, the necessary questions are: What is exactly endometritis and what are its reproductive consequences? When could a clinician start to diagnose an inadequate uterine involution? Which is the best diagnostic method to detect endometritis? Veterinary efforts have been focused on checking dairy cows at first week postpartum for metritis and after 21 days in milk for endometritis (Sheldon *et al.* 2006; Chapwanya *et al.* 2008; LeBlanc *et al.* 2008). To the best of our knowledge, no research has been made on days 15-21 postpartum to characterize clinical findings related to subsequent impaired fertility. Moreover, although endometrial biopsy is the gold standard for diagnosing the degree of uterus inflammation (Bonnett *et al.* 1991; Chapwanya *et al.* 2009), it is not a clinically used technique due to the difficulty of procedure and sample processing. Nowadays, other practices such as endometrial cytology or ultrasonography are being used to determine uterine health (Ginther 1998; Barlund *et al.* 2008, Kasimanickam *et al.* 2004, Gnemmi and Maraboli 2010).

Once postpartum uterus disease is detected, it is crucial to settle the best and faster therapeutic protocol. Hormone products are one of the daily used procedures to improve uterine health. Natural prostaglandin, due to its luteolytic and uterotonic activity has become a routine treatment during postpartum period (Rodriguez-Martinez *et al.* 1987; Pankowski *et al.* 1995). The aim of this treatment is to reduce bacterial and purulent content by uterine contractions and enhance uterine defense (Paisley *et al.* 1986). However, PGF_{2α} field trials reveal contradictory results that question its efficiency (Young and Anderson 1986; López-Gatius and Camón-Urgel 1989; Hendricks *et al.* 2006; Salasel and Mokhtari 2011). Antibiotic administration is another traditional treatment used after calving (Paisley *et al.* 1986). In contrast to systemic administration, intrauterine infusions achieve higher concentration of antibiotic on genital mucosa (Bretzlaff *et al.* 1983). Thus, several intrauterine protocols have been designed to solve uterine infection. For example, tetracycline (Goshen and Shpigel 2006), penicillin (Thurmond *et al.* 1993) and cephalosporin (Galvão *et al.* 2009) are three of the most common used drugs. The critical point in all these therapeutic procedures is the lack of a control group in many of them. As a result, contradictory outcomes can be observed and veterinary postpartum treatments cannot be easily compared (Burton and Lean 1995; Lefebvre and Stoc, 2012).

Finally, after the voluntary waiting period the cow has to conceive again. Fertility has been associated to management, environment or disease related factors (López-Gatius 2003, 2011; Garcia-Ispierto *et al.* 2007). For instance, management practices at this time have to be carefully performed to increase effectiveness of artificial insemination. One key point to improve fertility is the application of synchronization protocols (Larson and Ball 1992; De Rensis and Peters 1991; Wiltbank *et al.* 2011). These hormone combinations not only synchronize ovulations leading to a timed artificial insemination (Souza *et al.* 2009; Butler *et al.* 2011), but also can increase the estrous behavior and subsequent conception rate (Yániz *et al.* 2004; García-Ispierto *et al.* 2010,2012), specially during the warm season. There are two mainly types of protocols: based on PGF_{2α}, such as Ovsynch[®] (Pursley *et al.* 1997), and on progesterone-releasing intravaginal device. The main difference between these protocols is that the first one is more effective when cows are cyclic (Murugavel *et al.* 2003), and that progesterone protocols can also synchronize acyclic cows. Thus,

progesterone-based treatments can be proposed as a therapeutic protocol to synchronize all animals, but nowadays there is still no one single standard protocol to synchronize all kind of lactating dairy cows.

Progesterone based synchronizing protocols have been modified by adding hormones like gonadotrophin-releasing hormone (GnRH) or human chorionic gonadotrophin (hCG) in dairy cattle (DeRensis *et al.* 2002, 2010). Equine chorionic gonadotrophin (eCG) displays both FSH and LH-like activity (Bever *et al.* 1989, Papkoff 1998) and seems to enhance follicular growth and corpus luteum development (Rostami *et al.* 2011; Silva *et al.* 2002). Protocols combining PRID and eCG have only been tested in beef cattle, and frequently combined with estradiol, product not allowed in EU nowadays (Barruselli *et al.* 2003; Sá Filho *et al.* 2010).

In summary, this thesis scoped to improve reproductive efficiency of high producing dairy cows by controlling the postpartum period and implementing a new estrous synchronization protocol.

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MAIN OBJECTIVES

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- Characterize and define the early postpartum period in clinically healthy dairy cows in terms of adequate or inadequate uterine involution on the basis of methods such as ultrasonography, endometrial cytology and blood tests.
- Determine possible relationships between postpartum ultrasound findings of the genital tract on Days 15-21 postpartum and subsequent fertility in high producing dairy cows.
- Evaluate by transrectal ultrasonography the effect of a single prostaglandin $F_{2\alpha}$ dose on Day 15-21 postpartum on the subsequent conception rate.
- Evaluate by transrectal ultrasonography the effect of a routine postpartum practice established in a farm, based on a single intrauterine infusion of penicillin on cows suffering from endometritis on Days 15-21 postpartum.
- Evaluate by transrectal ultrasonography the effect of a single large-volume lavage with normal saline solution on cows suffering from endometritis on Days 15-21 postpartum.
- Determine whether the placement of a PRID followed by eCG treatment given on the day of device removal in cows at 51-57 days postpartum or in anoestrus period, after 120 days postpartum increases reproductive performance of high producing dairy cows.

CHAPTER 1

THE INFLUENCE OF GENITAL TRACT STATUS IN POSTPARTUM PERIOD ON THE SUBSEQUENT REPRODUCTIVE PERFORMANCE IN HIGH PRODUCING DAIRY COWS

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THE INFLUENCE OF GENITAL TRACT STATUS IN POSTPARTUM PERIOD ON THE SUBSEQUENT REPRODUCTIVE PERFORMANCE IN HIGH PRODUCING DAIRY COWS

Abstract

The aim of the present study was to characterize the early postpartum period in clinically healthy dairy cows by ultrasonography (US), endometrial cytology (EC) and white blood cell counts, and determine possible relationships between postpartum findings and subsequent reproductive performance. Fifty-three dairy cows were examined on Days 15-21 (Visit 1), 22-28 (Visit 2) and 29-35 (Visit 3) postpartum. The clinical exam included: examination of vaginal fluid, EC, rectal palpation and US of the genital tract (cervical diameter, endometrial thickness, presence of a corpus luteum (CL) or intrauterine fluid (IUF) and its echogenicity). Luteal activity (presence of a CL in a single visit), return to cyclicity (presence of a CL in two consecutive visits) and conception rate at 70 and 120 days postpartum were considered as the dependent variables in four consecutive binary logistic regression analyses. Factors affecting leukocyte counts were established by GLM repeated measures analysis of variance. Based on the odd ratio, the likelihood of luteal activity was higher in multiparous than primiparous cows (OR=3.75) and tended to diminish in cows showing increased endometrial thickness in Visit 1 (V1) (OR=0.06). The likelihood of returning to cyclicity decreased for each centimeter increase in cervical diameter in V1 (OR=0.14) and that of conception on Day 70 was lower in cows showing the presence of echogenic or anechogenic IUF in V1 (OR= 0.09 or OR= 0.13, respectively) compared to cows lacking IUF. Effects of parity and IUF were observed on neutrophil counts. Positive EC results were unrelated to the cumulative conception rate at 70 and 120 days in milk, whereas cows returning a positive EC result in V1 showed a greater likelihood of increased endometrial thickness. In conclusion, measuring cervical diameter, endometrial thickness and detecting the echogenicity of IUF by ultrasonography from Days 15 to 21 postpartum in clinically normal cows is an appropriate tool to predict subsequent reproductive performance. Vaginal examination and transrectal palpation alone did not emerge as valuable predictors.

Key words: endometritis, ultrasonography, intrauterine fluid, leukocyte counts, dairy cattle.

1. INTRODUCTION

Although dairy cattle have shown a continuous increase in milk production over the years, their fertility has suffered a dramatic decline. The reasons for this decline are multifactorial and not only attributable to increased milk production (Lucy 2001; López-Gatius 2003). The postpartum period has been recognized as a critical event in the reproductive performance of dairy cattle (LeBlanc 2008). In fact, parturition is a considerable welfare risk in high producing dairy cows and especially during this period veterinarian visits focus on trying to improve the reproductive efficiency of cows (Sheldon and Dobson 2004; Sheldon *et al.* 2008; Salasel *et al.* 2010). After parturition, the cow has both to undergo adequate uterine and cervix involution, and return to ovarian cyclicity (Morrow *et al.* 1969a). Despite the impact of the postpartum period (Louca and Legates 1967; LeBlanc 2008) on herd economy, we still lack a clear definition of what adequate uterine involution involves (Sheldon *et al.* 2006), especially before finishing the voluntary waiting period.

Traditionally, rectal palpation and vaginal examination have been used to determine the reproductive status of the postpartum. However, these methods are too subjective and may underestimate the incidence of uterine disorders (Barlund *et al.* 2008). Moreover, most postpartum uterine disorders are related to endometrial inflammation (Gilbert *et al.* 2005), which is usually subclinical. Thus, traditional palpation needs complementing with more precise and objective techniques (Gilbert 1992; Kasimanickam *et al.* 2004; Westermann *et al.* 2010). Transrectal ultrasonography (US) is a rapid method that gives information about intrauterine contents, endometrial thickness and cervical size, which are variables closely related to uterine involution (Okano and Tomizuka 1987; Kasimanickam *et al.* 2004; Barlund *et al.* 2008). In addition, obtaining blood samples to determine white blood cells could be a useful diagnostic tool during the postpartum period since the immune response of the cow is decisive for overcoming postpartum bacterial contamination (Mateus *et al.* 2002a; Nazifi *et al.* 2008). Finally, in clinical examinations endometrial cytology has been used

to diagnose subclinical endometritis (Kasimanickam *et al.* 2004; Westermann *et al.* 2010) from Day 20 postpartum. Subclinical endometritis diagnosed by uterine cytology seems to be related to impaired reproductive performance (Salasel *et al.* 2010) although this relationship is still unclear (Plöntzke *et al.* 2010). However, endometrial involution starts much earlier. Endometrial changes are needed to return to previous non-pregnant status. Epithelial regeneration is complete by about 25 days after parturition, although complete histological regression finished around 6-8 weeks postpartum (Sheldon *et al.* 2008). Cytology may be an appropriate indicator of an adequate postpartum course. Hence, cytology and ultrasonography could help clinicians detect cows with inadequate uterine involution even before 20 days postpartum.

Puerperal diseases such as metritis or endometritis disrupt adequate postpartum involution and substantially impair reproductive performance (LeBlanc *et al.* 2002; LeBlanc 2008). Uterine infections not only damage the uterus, they also suppress GnRH and LH secretion and have localized effects on ovarian function (Sheldon and Dobson 2004). Consequently, ovarian function will be intimately related to subsequent fertility (Stevenson and Call 1983; Galvaõ *et al.* 2010) and to minimize further reproductive complications, uterine involution and resumption of ovarian cyclicity will have to be carefully assessed.

The objectives of our study were: (1) to characterize and define the early postpartum period in clinically healthy dairy cows in terms of adequate or inadequate uterine involution on the basis of methods such as ultrasonography, endometrial cytology and blood tests, and (2) to determine possible relationships between postpartum findings and subsequent fertility.

2. MATERIAL AND METHODS

2.1. Cattle and herd management

The study was performed on a commercial herd comprising 650 high-producing Holstein-Friesian dairy cows in northeastern Spain. The study population included 53 clinically normal postpartum dairy cows that had calved from October 2009 to April 2010. Herd management involved the use of pedometers, housing in free stalls with

concrete slatted floors and cubicles, daily postpartum checks and a weekly reproductive health program.

The mean annual culling rate and milk production during the study period were 28% and 10.650 kg / cow, respectively. The cows were grouped according to age (primiparous vs multiparous), milked three times daily and fed complete rations. Feeds consisted of cotton seed hulls, barley, corn, soybean, bran and roughage, primarily corn, barley or alfalfa silage and alfalfa hay. Rations were in line with NRC recommendations (NCR 2001). Dry cows were kept in a separate group and transferred, depending on their body condition score and age, 7–25 days prior to parturition to a ‘parturition group’. An early postpartum, or ‘fresh cows’, group was also established for postpartum nutrition and control. Clinically normal cows were transferred to separate groups from 7 days postpartum onwards. Clinically normal animals are defined as cows free of diseases such as lameness, mastitis, digestive disorders and puerperal metritis.

2.2. Reproductive management

In postpartum daily checks, the following puerperal diseases were treated until resolved or until culling: signs of injury to the genital area (i.e. vaginal or recto-vulvar lacerations), metabolic diseases such as hypocalcaemia and ketosis (the latter, diagnosed during the first or second week postpartum), retained placenta (fetal membranes retained longer than 12 h after parturition) or primary metritis (diagnosed during the first or second week postpartum in cows not suffering placental retention). Cows with a retained placenta or primary metritis were always treated with oxytetracycline boluses introduced into the uterus.

The voluntary waiting period for the herd was 50 days in milk. Cows from 51 to 57 d postpartum with no uterine or ovarian disorders detected by ultrasound were fitted with a progesterone releasing intravaginal device (PRID, containing 1.55 g of progesterone, CEVA Salud Animal, Barcelona, Spain) left for 9 days. These animals were also given prostaglandin F_{2α} (25 mg dinoprost i.m.; Enzaprost, CEVA Santé Animale, Libourne, France) 24 h before PRID removal (García-Ispuerto *et al.* 2010). Moreover, estrus was detected by pedometers and confirmed by palpation per rectum at AI (López-Gatius and Camón-Urgel 1991). All animals were artificially inseminated

with frozen semen from bulls of proven fertility. If cows returned to estrus, their status was confirmed by ultrasound, and the animals were recorded as non-pregnant. In the remaining cows, pregnancy diagnosis was performed by ultrasound 28–34 days post-insemination.

2.3. Clinical examination

During the scheduled herd visit, an exam was performed on Days 15-21 (V1), 22-28 (V2) and 29-35 (V3) postpartum by the same veterinarian. Cows receiving systemic antibiotic therapy, intrauterine therapy or reproductive hormone administration in the current lactation, or had abnormal internal genitalia including adhesions, lacerations and/or pyometra within seven days prior to V1, were excluded from the study. The clinical exam during the three visits included rectal palpation and ultrasonography (US) of the genital tract, examination of vaginal fluid and endometrial cytology.

2.3.1 Rectal palpation and ultrasonography of the reproductive tract

According to uterine location (abdominal or pelvic), contents, tone, mobility and horn asymmetry examined by rectal palpation, uterine involution was classified as adequate, regular, or inadequate. Further, the entire reproductive tract was examined by ultrasound using a portable B-mode ultrasound scanner (Easi-scan with a 7.5 MHz transducer). Scanning was performed carefully and slowly along the dorsal/lateral surface of cervix and each horn through the ovaries. Cranial cervical size and endometrial thickness were measured using the internal calipers of the ultrasonograph, and intrauterine fluid (IUF) was scored as absent, anechogenic or echogenic fluctuant or compact contents (Figure 1). The presence of a corpus luteum (CL) in one or both ovaries was also recorded.

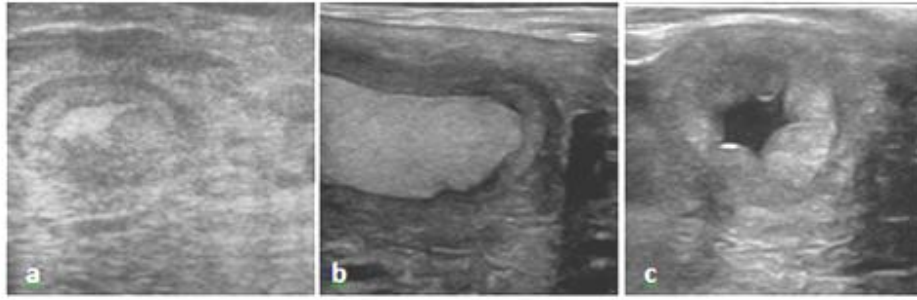


Figure 1. Cross-sectional images of the uterine horns showing compact echogenic (a), fluctuant echogenic (b) or anechogenic (c) intrauterine fluid.

2.3.2 Vaginal fluid examination and endometrial cytology

The vulva was cleaned using a wet paper towel and then a clean lubricated gloved hand was inserted through the vulva into the vagina to withdraw the vaginal contents for examination. Each time, the vaginal discharge was scored qualitatively as clear fluid, predominately clear fluid with some flecks of pus, mucopurulent (approximately 50% pus and 50% mucus), or purulent (>50% pus) (according to the vaginal discharge score of Williams (Williams *et al.* 2005). Vaginal fluid odor was classified as present or absent. Manual vaginal examination was only performed in V1.

The cytobrush (Cell sampler, Deltalab S.L.U., Barcelona, Spain) used to obtain endometrial samples was 20 mm in length and 6 mm in diameter. It was adapted to a uterus culture swab with a 50-cm long pipette inside (Minitube, Tiefenbach, Germany) and protected by a second inner sheath. Briefly, the vulva was cleaned using a wet paper towel and the device inserted via the cervix into the uterine body. Inside the uterus, the external sheath was retracted, and the brush was pushed gently forward and rolled across the uterine wall. Thereafter, the brush was retracted into the inner sheath to prevent contamination during passage through the genital tract. The brush was then rolled onto a sterile glass microscopy slide. The slide was immediately fixed and stained using a modified Giemsa stain (Fast panoptic, Panreac, Barcelona, Spain). Three hundred cells were counted under a microscope (x400 magnification) to determine the proportion of polymorphonuclear leukocytes (PMN). The threshold value used for the proportion of PMN indicating subclinical endometritis was 18% on Days 20-33 (V2) and 10% on Days 34-47 (V3) (Kasimanickam *et al.* 2004). However, since there is no

reference providing the threshold for Days 15-21 (V1) and it is difficult to differentiate the normal uterine involution and the subclinical endometritis, we considerer citologies in the highest quartile of PMNN percentage as inadequate uterine involution. The endometrial cytology was scored as positive when the established threshold was exceeded.

2.4 Blood sampling

Blood samples were collected from the coccygeal vein into heparinized vacuum tubes (BD Vacutainer™, Becton, Dickenson and Company, Plymouth, UK) in 46 cows. Total leukocyte counts were performed using an automated blood analyzer (Hemavet Cell counter 850, CDC Technologies, Inc., Centerville, USA) standardized for the analysis of cow's blood. Total leukocyte numbers, and numbers of neutrophils and lymphocytes were expressed as counts per microliter of blood.

2.5. Data Collection and analysis

The variables recorded for each cow were: parity (primiparous vs multiparous), type of parturition (normal- or dystotic parturition), stillbirth, previous twin pregnancy, retention of the placenta (retention of the fetal membranes >24 hours), puerperal metritis, hypocalcemia, abomasal displacement, ketosis, lameness and/or clinical mastitis before or at first postpartum examination; CL presence, measures of the cervix and endometrium (cm), endometrial cytology and vaginal content and odor; AI date, semen providing bull, AI technician and conception rate for cows inseminated before Days 70 and 120 in milk.

Luteal activity was defined as the presence of a single CL during the study period. A cow was recorded as having returned to cyclicity when a CL was detected in two consecutive examinations. Given the low incidence of disorders such as dystotic parturition, stillbirth, previous twin pregnancy and placenta retention, these were pooled into one category (peripartum disorders) and coded as a dichotomous variable where 1 denotes presence and 0 denotes absence. Parity, luteal activity and return to cyclicity were also coded as dichotomous variables. Vaginal contents (clear fluid, predominately clear fluid with some flecks of pus, mucopurulent, or purulent) and odor (not present or

present), IUF (absent, anechogenic or echogenic fluid) and uterine involution (adequate, regular or inadequate) were considered as class variables. Uterine and cervix measurements, total and differential leukocyte counts were considered continuous variables.

Dichotomous and class variables from V1 to V3 were compared within visits using Fisher's exact test. The Student t-test was used to compare mean values of continuous variables among visits. Positive endometrial cytology rates and conception rate before 70 and 120 d postpartum were compared using the Chi-squared test and mean endometrial measurements and cytology results in each visit were compared by ANOVA.

Four binary logistic regression analyses were performed. The corresponding dependent variables were luteal activity, return to cyclicity and conception rate at 70 or 120 d in milk, and the above mentioned V1 variables were considered as independent variables. Logistic regression analyses were performed using the SPSS package, version 15.0 (SPSS Inc., Chicago, IL, USA) according to the method of Hosmer and Lemeshow (Hosmer and Lemeshow 1989). Basically, this method involves five steps as follows: preliminary screening of all variables for univariate associations; construction of a full model using all variables found to be significant in the univariate analysis; stepwise removal of non-significant variables from the full model and comparison of the reduced model with the previous model for model fit and confounding; evaluation of plausible interactions among variables; and assessment of model fit using Hosmer–Lemeshow statistics. Variables with univariate associations showing P values <0.25 were included in the initial model. We continued modeling until all the main effects or interaction terms were significant according to the Wald statistic at P <0.05.

The effects of parity and presence of IUF, on blood tests (total leukocyte, lymphocyte, and neutrophil counts) from Days 15-21 to 29-35 postpartum were evaluated by GLM repeated measures analysis of variance using the SPSS computer package, version 15.0 (SPSS Inc., Chicago, IL, USA) (three GLM repeated measures analyses). Analysis of variance (ANOVA) was used to compare significant variables of GLM repeated measures analysis at each time point.

3. RESULTS

The mean lactation number and daily milk production at 53 d postpartum were 1.6 ± 0.78 lactations (mean \pm S.D.; range: 1-4 lactations) and 43.7 ± 7.12 Kg (range: 26.1 - 64.4 Kg), respectively. Forty four cows (83%) had a normal delivery with no clinical complications, whereas 9 (17%) cows suffered a peripartum disorder. Table 1 shows the prevalence of clinical findings on examination. Uterine involution as assessed by rectal palpation improved significantly during the study period. The percentage of cows that had intrauterine fluid detected by ultrasonography decreased during subsequent weekly exams. Further, compact echogenic IUF could not be detected by rectal palpation. Thus, this kind of echogenic IUF could only be detected by ultrasound. Cervical diameter decreased significantly and endometrial thickness did not change over time. Total leukocyte counts as well as lymphocyte and neutrophil counts all remained unchanged during the study period.

Table 1. Percentages and means of reproductive and blood variables determined in visits conducted on Days 15-21 (V1), 22-28 (V2) and 29-35 (V3) postpartum.

Clinical findings	V1*	V2*	V3*
<i>Class variables</i>			
Intrauterine fluid (N: 53)			
Absent	10 (18.9) ^a	28 (52.8) ^b	27 (50.9) ^b
Anechogenic	30 (56.6) ^a	18 (34) ^b	24 (45.3) ^{a,b}
Echogenic	13 (24.5) ^a	7 (13.2) ^{a,b}	2 (3.8) ^b
Uterine involution (N: 53)			
Adequate	22 (41.5) ^a	30 (56.6) ^a	47 (88.7) ^b
Regular	26 (49.1) ^a	22 (41.5) ^a	6 (11.3) ^b
Inadequate	5 (9.4)	1 (1.9)	0 (0)
<i>Continuous variables</i>			
Cervical diameter (cm) (N: 53)	3.6 ± 0.6 (2.5-7) ^a	3.1 ± 0.3 (2.5 - 4) ^b	2.9 ± 0.3 (2.5 - 4) ^b
Endometrial thickness(cm) (N:53)	0.6 ± 0.2 (0.3 - 1.3)	0.6 ± 0.2 (0.3 - 1)	0.5 ± 0.2 (0.3 - 1)
Blood variables ($\times 10^3/\mu\text{l}$) (N: 46)			
Total leukocyte count	7.7 ± 1.7 (4.4 -13.9)	7.8 ± 1.7 (4.6-11.3)	7.3 ± 1.4 (4.2 - 10.3)
Neutrophil count	4.3 ± 1.3 (1.9 - 9.2)	4.3 ± 1.7 (1.8 - 8.4)	4.1 ± 1.2 (2.1 - 7.2)
Lymphocyte count	2.1 ± 0.7 (0.9 - 4.8)	2 ± 0.6 (0.5 - 4.2)	2.1 ± 0.7 (1 - 3.8)

^{a, b} Within a row, values with a different superscript were significantly different ($p < 0.05$)

* n (%) provided for class variables and mean \pm SD for continuous variables.

Twenty (37.7%) of the animals examined had transparent vaginal fluid, 19 (35.8%), 11 (20.8%) and 3 (5.7%) animals had some flecks of pus, mucopurulent, or purulent fluid, respectively. Only 7.5% had vaginal fluid odor. Table 2 shows the endometrial cytology results recorded in V1 and the percentages recorded in subsequent examinations. Positive cytology results were not related to cumulative conception rates at 70 and 120 days in milk, whereas cows showing a positive endometrial cytology in V1 were more likely to show increased endometrial thickness. No significant correlation was detected in subsequent examinations (V2 and V3).

Twenty seven cows became cyclic (50.9%): 13.2%, 35.8% and 50.9% in V1, V2 and V3, respectively. The conception rate on Day 120 postpartum for cows inseminated during this period was 58.8%.

Table 2. Endometrial cytology results over the postpartum period

First visit* (15-21 days postpartum)	Subsequent visits* (22-35 days postpartum)	n (%) [†]
Negative (n=36)	Remained negative	20 (43.5)
	Changed to positive	16 (34.8)
Positive (n=10)	Remained positive	4 (8.7)
	Changed to negative	6 (13)

*A cytology was considered positive when the percentage of PMN was equal or higher than highest quartile, 18 or 10 on first, second or third examination respectively.

[†] Percentages are referred to the total number of cows.

The first binary logistic regression analysis indicated no significant effects of peripartum events, vaginal fluid and odor score, IUF, cervical diameter and uterine involution in V1 on luteal activity. No plausible significant interactions were found. Based on the odds ratio, the likelihood of luteal activity increased in multiparous compared to primiparous cows (by a factor of 3.75; $p=0.03$). Cows showing increased endometrial thickness in V1 tend to be less likely to exhibit luteal activity (by a factor of 0.06, $p=0.052$) (Table 3).

Table 3. Odds ratios of the variables included in the final logistic regression model for factors affecting luteal activity.*

Factor	Class	n (%)	Odds ratio	95% CI	P - value
Parity	Primiparous	9/27 (33%)	Reference		
	Multiparous	18/26 (69%)	3.75	1.12-12.52	0.031
Endometrial thickness V1 [†]	Continuous	53	0.06	0.01-1.02	0.052

R. Nagelkerke= 0.254. P value for the model 0.004.

CI, confidence interval for the odds ratio.

* Represented by cows showing the presence of a corpus luteum in at least one examination.

† Endometrial thickness measured on Days 15-21 postpartum.

The second binary logistic regression revealed no significant effects of parity, peripartum disorders, vaginal fluid and odor score, IUF, uterine involution and endometrial thickness in V1 on return to cyclicity. No plausible significant interactions were found. Based on the odds ratio, the likelihood of returning to cyclicity decreased for each centimeter increase in cervical diameter in V1 by a factor of 0.14 (Table 4).

Table 4. Odds ratios of the variables included in the final logistic regression model for factors affecting return to cyclicity.*

Factor	Class	n	Odds ratio	95% CI	P – value
Cervical diameter V1 *	Continuous	53	0.14	0.03-0.76	0.02

R Nagelkerke= 0.189. P-value for the model 0.006

CI, confidence interval for the odds ratio.

* Represented by cows showing the presence of a corpus luteum in two consecutive examinations.

† Cervical diameter measured on Days 15-21 postpartum.

Finally, no significant effects were detected of parity, peripartum disorders, vaginal fluid and odor score, uterine involution, endometrial thickness and cervical diameter in V1, semen-providing bull and AI technician on the likelihood of conception in cows inseminated before Day 70 postpartum. No plausible significant interactions were found. Based on the odds ratio, the likelihood of conception on Day 70 was lower in cows showing the presence of echogenic or anechogenic IUF in V1 (by factors of 0.09 or 0.13, respectively) compared to cows not showing the presence of IUF (Table 5). None of the variables above affected the conception rate on Day 120 postpartum.

Table 5. Odds ratios of the variables included in the final logistic regression model for factors affecting conception rate at 70 days in milk (n= 51)

Factor	Class	n (%)	Odds ratio	95% CI	P – value
FIU*	Absent	7/9 (77.8%)	Reference		
	Anechogenic	9/29 (31%)	0.13	0.02-0.75	0.02
	Echogenic	3/13 (37.3%)	0.09	0.01-0.66	0.02

R Nagelkerke= 0.195. P value for the model 0.02.

CI, confidence interval for the odds ratio.

* Intrauterine fluid evaluated on Days 15-21 postpartum

Through GLM repeated measures analysis, no significant effects were observed of parity and presence of IUF on total leukocyte counts in all the postpartum examinations.

Table 6. Main model of the GLM repeated measures analysis of factors affecting neutrophil counts throughout the postpartum period

Subject effect	Factor	Df	F	P
Between	Parity	1	5.00	0.031
	IUF	2	4.82	0.013

Mauchly's sphericity = 0.044

IUF, intrauterine fluid

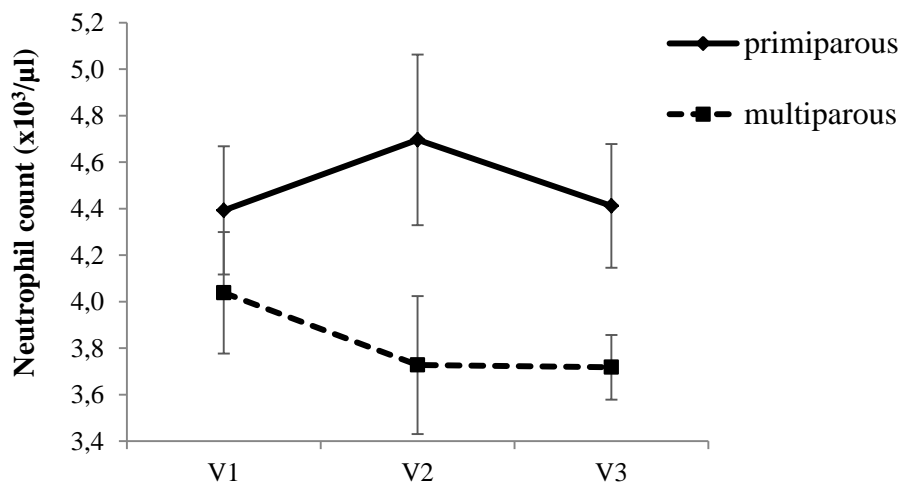


Fig2. Mean neutrophil counts (cells 10³µl ±SD) recorded in the postpartum visits (V1: Day 15-21, V2: Day 22-28, V3: Day 29-35) in primiparous (n=26) and multiparous cows (n=20). (*significant differences -p<0.05- between the means of repeated measures determined by analysis of variance ANOVA).

A second GLM analysis indicated significant effects of parity and IUF (between subject effects) on neutrophil counts throughout the study period. Analysis of variance (ANOVA) showed significant effects of parity and IUF on V2 and V3. Table 6 provides the variables affecting neutrophil counts remaining in the final model. Figures 2 and 3 provide descriptive values for neutrophil counts recorded throughout the study period in primiparous and multiparous cows and in cows with IUF.

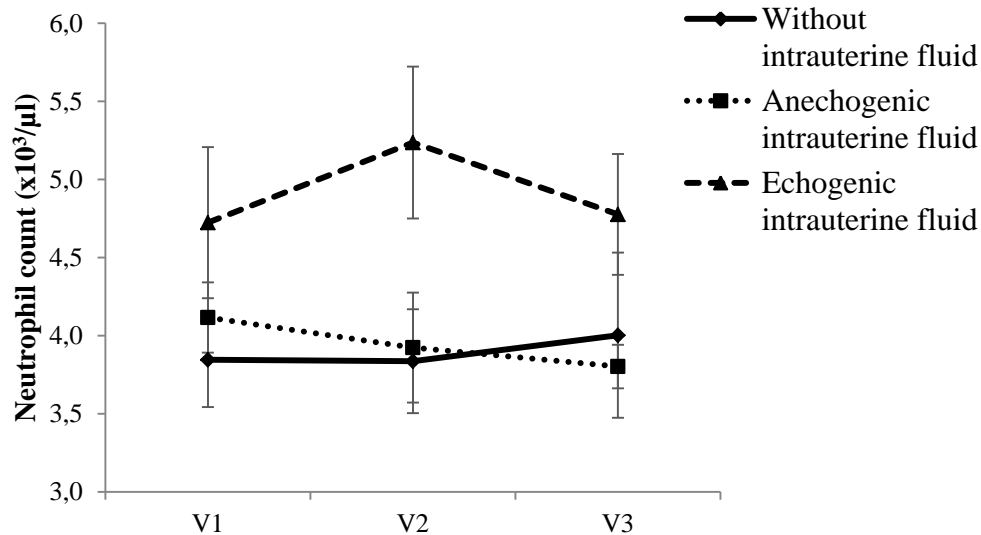


Fig3. Mean neutrophil counts (cells $10^3 \times \mu\text{l} \pm \text{SD}$) recorded in the postpartum visits (V1: Day 15-21, V2: Day 22-28, V3: Day 29-35) in lactating dairy cows showing the presence (echogenic -n=13- or anechogenic -n=24-) or absence (n=9) of intrauterine fluid. (*significant differences - $p < 0.05$ - between the means of repeated measures determined by analysis of variance ANOVA).

Finally, no effects were found of any of the variables considered in the model on lymphocyte counts throughout the postpartum period.

4. DISCUSSION

In this study, we were able to identify clinical findings from Day 15-21 postpartum that could be considered to be predictors of subsequent reproductive performance in high producing dairy cows. Through ultrasonography, compact echogenic IUF can be detected and differentiated from anechogenic fluid. The presence of IUF, regardless of its echogenicity, has been related to subsequent fertility (Kasimanickam *et al.* 2004; Dourey *et al.* 2011). Bacterial contamination of the uterus triggers an inflammatory response followed by purulent fluid secretion into the uterine

lumen (Frank *et al.* 1983). Thus, it could be that the echogenicity of IUF reflects the extent of inflammation. In fact, presence of echogenic IUF was related to higher neutrophil counts during examinations compared with cows without IUF. In our study, cows with echogenic or anechogenic IUF on Days 15-21 postpartum were 11.1 (1/0.09) or 7.7 times (1/0.13) less likely to be pregnant on Day 70 postpartum than cows lacking IUF, respectively. Thus, the focus of postpartum attention should be earlier, during the early postpartum. In this way, cows would reach this stage showing adequate involution. In effect, by Day 120 postpartum the pregnancy rate was unaffected, probably because of the complete recovery of cows with prior IUF.

Cows with a greater cervical diameter measured in the first visit (V1) conducted on Days 15-21 postpartum were less likely to resume ovarian cyclicity than cows with a smaller diameter. Delayed postpartum cyclicity has a great impact on herd economy (Inchaisri *et al.* 2010). However, measures for the early detection of these non-cyclic cows have not yet been established. For example, several authors have differently correlated cervix involution with subsequent reproductive performance (LeBlanc *et al.* 2002; Kasimanickam *et al.* 2004; Senosy *et al.* 2009). Our results highlight the existing controversy over the relationship between cervix diameter and reproductive performance. The cervix and endometrial mucosa undergo intimate crosstalk during involution (Morrow *et al.* 1969b) such that larger cervical diameters associated with uterine inflammation can give rise to abnormal follicular selection and abnormal ovarian cycles (Williams *et al.* 2007).

Endometrial thickness has been used as a diagnostic tool for endometritis [10]. In this study, although we only observed a tendency ($p=0.052$), clinically normal cows with a thicker endometrium in V1 showed delayed ovarian cyclicity compared to their partners. During the first week postpartum, a peripheral leukopenia exists related to the migration of leukocytes towards the uterine lumen (Mateus *et al.* 2002b; Nazifi *et al.* 2008). Although this endometrial leukocyte infiltration is essential to resolve postpartum uterine contamination (Singh *et al.* 2008), this physiological inflammation could result in endometritis or (Sheldon *et al.* 2006; Singh *et al.* 2008) affecting the resumption of ovarian cyclicity (El-Din Zain *et al.* 1995). On the other hand, endometritis can also be assessed by an endometrial cytology (Kasimanickam *et al.* 2004; Gilbert *et al.* 2005; Dubuc *et al.* 2010). In the present study, a positive

endometrial cytology result was associated with endometrial thickness on Day 15-21 postpartum, which suggests that inadequate uterine involution can be detected by ultrasonography. In future work, more animals will be needed to confirm that on Days 15-21 postpartum, ultrasonography is the easiest technique to detect abnormal uterine involution in healthy animals.

Both vaginal and rectal examinations have been widely reported as optimal methods for diagnosing puerperal metritis or endometritis (Gilbert 1992; Opsomer *et al.* 2000; LeBlanc 2002; Sheldon *et al.* 2006). However, in some animals the cervix rapidly closes after parturition and this prevents the expulsion of purulent fluid. Besides, rectal palpation does not serve to assess uterine features such as endometrial thickness or the echogenicity of IUF. In our study, we could not identify relationships between vaginal discharge or rectal palpation findings in V1 and subsequent reproductive performance.

Finally, multiparous cows were more likely to return to cyclicity than primiparous cows, in agreement with the findings of other studies (Opsomer *et al.* 2000; Santos *et al.* 2009). An effect of parity was also shown on white blood cell numbers. Thus, primiparous cows had higher neutrophil counts than multiparous cows, as previously reported (Mehrzaad *et al.* 2002). On the contrary, peripartum events had no effects on cyclicity or conception rate, probably due to good postpartum management practices conducted on the farm.

In conclusion, our results indicate that measuring cervical diameter, endometrial thickness and detecting the echogenicity of IUF by ultrasonography from Days 15 to 21 postpartum is a good tool to predict subsequent reproductive performance in clinically normal dairy cows. Conversely, vaginal examination and transrectal palpation alone are not valuable predictors of subsequent reproductive performance.

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CHAPTER 2

THERAPY HORMONAL TREATMENT FOR ABNORMAL UTERINE INVOLUTION ON HIGH PRODUCING DAIRY COWS.

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THERAPY HORMONAL TREATMENT FOR ABNORMAL UTERINE INVOLUTION ON HIGH PRODUCING DAIRY COWS.

Abstract.

The aim of this study was to evaluate by transrectal ultrasonography the effect of a single Prostaglandin F_{2α} (PG) dose on Day 15-21 postpartum on the subsequent conception rate. Abnormal uterine involution and the presence or absence of corpus luteum at the moment of treatment were considered as factors. One hundred forty cows were examined on Days 15-21 (V1), 22-28 (V2) and 29-35 (V3) postpartum. Cranial cervical size, endometrial thickness, presence of intrauterine fluid (IUF) and corpus luteum (CL) were recorded by transrectal ultrasonography. Cows considered as suffering endometritis included the following criteria: presence of echogenic IUF, cervical diameter of ≥ 4 cm or endometrial thickness of ≥ 0.75 cm. Cows were randomly assigned to a control (n= 87) or PG treatment group (n=53) (receiving a single dose of PG on V1) and classified as presenting abnormal (presence of echogenic IUF or increased genital tract measures) or normal uterine findings in V3. Cox proportional-hazards regressions were performed to determine factors affecting subsequent conception rate of cows with endometritis in V1. No significant effect of PG treatment on conception rate was observed. Based on the hazard ratio, the likelihood of pregnancy was lower in multiparous and in cows with abnormal uterine findings in V3 compared with the remaining cows (HR=0.56 and 0.35, respectively). In cows suffering of echogenic IUF without cervical or endometrial enlargement, the likelihood of pregnancy was higher in cyclic cows in V1 (HR= 2.29). Chi-square test revealed no relationship between abnormal uterine findings in V3 and group of treatment in V1. In conclusion, no effect of treatment with PG on Days 15-21 postpartum was observed on subsequent reproductive performance of high producing dairy cows, even in cyclic animals. Irrespective of treatment, early resumption of ovarian cyclicity enhances postpartum recovery and improves subsequent conception rate.

Keywords: transition period, bovine, genital tract, puerperium, metritis, fertility, ultrasound

1. INTRODUCTION

Postpartum uterus disease has been proved to impair subsequent reproductive performance in cattle (Dobson *et al.* 2007; Le Blanc 2008). Bacterial uterus contamination after parturition can compromise local uterine immunology and induce uterine disease (Frank *et al.* 1983; Singh *et al.* 2008). Although many studies have been focused on preventing and treating postpartum uterine disorders in dairy cows, characterization of clinical or subclinical endometritis are still under discussion (Sheldon *et al.* 2006; Le Blanc 2008; Dubuc *et al.* 2010). Uterine biopsy is the most accurate technique to diagnose endometritis (Bonnet *et al.* 1991; Chapwanya *et al.* 2009), but usually this type of diagnoses escape from clinical level. Cytological procedures are more commonly used to identify healthy cows with inflammation of endometrium (Kasimanickam *et al.* 2004; Barlund *et al.* 2008; Dourey *et al.* 2010). Transrectal ultrasonography has also successfully been used to detect abnormal uterine involution (Ginther 1998; DesCôteaux *et al.* 2009) and to recognize animals with an impaired fertility (Kasimanickam *et al.* 2004; López-Helguera *et al.* 2012). Measuring cervical diameter, endometrium thickness and detecting intrauterine fluid (IUF) can help clinicians to identify subsequent subfertile cows (López-Helguera *et al.* 2010, 2012).

A wide range of treatments have been described in postpartum cows, but there is still no gold standard protocol to overcome endometritis (Burton and Lean 1995; Lefebvre and Stock 2012). Antibiotics and hormonal products are basically the two traditional therapeutic options. Prostaglandin F_{2α} (PG) is the most used hormone along postpartum period (Burton and Lean 1995). Natural prostaglandins have both, luteolytic and uterotonic effect that could improve bacterial uterus elimination (Pankowski *et al.* 1995). Whether the cow is cyclic, the PG may also induce a new ovarian follicular wave, enhancing animal immune response due to the immunostimulating effects of estradiol (Roth *et al.* 1983; Lewis 2004). Despite of this, there are still conflicting reports on the effectiveness of PG application regarding subsequent reproductive performance (Young and Anderson 1986; López-Gatius and Camón-Urgel 1989; Kasimanickam *et al.* 2005; Hendricks *et al.* 2006) because there is not an established therapeutical protocol.

For all that reasons, the aim of this study was to evaluate by transrectal ultrasonography the effect of a single Prostaglandin F_{2α} (PG) dose on Day 15-21 postpartum on the subsequent conception rate.

2. MATERIAL AND METHODS

2.1. Cattle and herd management

The study was performed on a commercial herd comprising 650 high-producing Holstein-Friesian dairy cows in northeastern Spain. The study population included 140 clinically normal postpartum dairy cows that had calved from October 2009 to November 2011. Herd management involved the use of pedometers, housing in free stalls with concrete slatted floors and cubicles, daily postpartum checks and a weekly reproductive health program.

The mean annual culling rate and milk production during the study period were 28% and 10.650 kg / cow, respectively. The cows were grouped according to age (primiparous vs multiparous), milked three times daily and fed complete rations. Feeds consisted of cotton seed hulls, barley, corn, soybean, bran and roughage, primarily corn, barley or alfalfa silage and alfalfa hay. Rations were in line with NRC recommendations (NRC 2001). Dry cows were kept in a separate group and transferred, depending on their body condition score and age, 7–25 days prior to parturition to a ‘parturition group’. An early postpartum, or ‘fresh cows’, group was also established for postpartum nutrition and control. Clinically normal cows were transferred to separate groups from 7 days postpartum onwards. Clinically normal animals are defined as cows free of diseases such as lameness, mastitis, digestive disorders and puerperal metritis.

2.2. Reproductive management

In postpartum daily checks, the following puerperal diseases were treated until resolved or until culling: signs of injury to the genital area (i.e. vaginal or recto-vulvar lacerations), metabolic diseases such as hypocalcaemia and ketosis (the latter, diagnosed during the first or second week postpartum), retained placenta (fetal membranes retained longer than 12 h after parturition) or primary metritis (diagnosed during the

first or second week postpartum in cows not suffering placental retention). Cows with a retained placenta or primary metritis were always treated with oxytetracycline boluses introduced into the uterus.

The voluntary waiting period for the herd was 50 days in milk. Cows from 51 to 57 d postpartum with no uterine or ovarian disorders detected by ultrasound were fitted with a progesterone releasing intravaginal device (PRID, containing 1.55 g of progesterone, CEVA Salud Animal, Barcelona, Spain) left for 9 days. These animals were also given prostaglandin F_{2α} (25 mg dinoprost i.m.; Enzaprost, CEVA Santé Animale, Libourne, France) 24 h before PRID removal (García-Ispuerto *et al.* 2010). Moreover, estrus was detected by pedometers and confirmed by palpation per rectum at AI (López-Gatius and Camon-Urgel 1991). All animals were artificially inseminated with frozen semen from bulls of proven fertility. If cows returned to estrus, their status was confirmed by ultrasound, and the animals were recorded as non-pregnant. In the remaining cows, pregnancy diagnosis was performed by ultrasound 28–34 days post-insemination. Fetal loss was recorded when the 90–96 days-diagnosis proved negative.

2.3. Clinical examination

During the weekly herd reproductive visit, a transrectal ultrasonography exam was performed on Days 15-21 (V1), 22-28 (V2) and 29-35 (V3) postpartum by the same veterinarian. The entire reproductive tract was examined by ultrasound using a portable B-mode ultrasound scanner (Easi-scan with a 7.5 MHz transducer). Scanning was performed along the dorsal/lateral surface of each horn and cervix. Cranial cervical size and endometrial thickness were measured using the internal calipers of the ultrasonograph, and intrauterine fluid (IUF) was scored as absent, anechogenic or echogenic as previously reported (López-Helguera *et al.* 2012). The presence of a corpus luteum (CL) in one or both ovaries was also recorded.

2.4. Treatment

Based on previous experimental findings (López-Helguera *et al.* 2010, 2012), cows considered as suffering endometritis included the following criteria: presence of echogenic IUF, cervical diameter of ≥ 4 cm or endometrial thickness of ≥ 0.75 cm. Cows

receiving reproductive hormone administration, systemic antibiotic or intrauterine therapy in the current lactation within seven days prior to V1, were excluded from the study. Cows were randomly into a control (n= 87) and a PG group (n=53). Animals in the treatment group were given a single dose of PG (25 mg dinoprost i.m.; Enzaprost, CEVA Santé Animale, Libourne, France) in V1. Cows were reexamined on the following weeks by ultrasonography. In case of presence of echogenic IUF on V1, IUF disappearance or permanence was evaluated in V2. When echogenic IUF persisted or abnormal uterine findings were observed on V3, a second PG treatment was administrated.

2.5. Data Collection and analysis

The variables recorded for each cow were: parity (primiparous vs multiparous), season of parturition (warm –May to September- vs cool -October to April) (Labèrnia *et al.* 1998; López-Gatius 2003), type of parturition (normal vs dystotic), stillbirth, previous twin pregnancy, retention of the placenta (retention of the fetal membranes >12 hours), antibiotic treatment on first week after calving (presence or absence), treatment group (0=control vs 1=PG), CL presence in V1, abnormal uterine findings on V3 (presence or absence of echogenic IUF), date of conception, days open and early pregnancy loss (<90 days of pregnancy).

Previous disorders such as dystotic parturition, stillbirth, previous twin pregnancy and placenta retention, were pooled into one single category (peripartum disorders) and coded as a dichotomous variable where 1 denotes presence and 0 denotes absence. Parity, season of parturition, treatment group, CL presence at V1, previous treatment, abnormal uterine findings on V3, IUF disappearance and fetal loss following pregnancy diagnosis were also coded as dichotomous variables.

All statistics procedures were performed using the SPSS package version 18.0 (SPSS 20 Inc., Chicago, IL, USA) with the level of significance set at $P < 0.05$. Associations between treatment group, abnormal uterine findings on V3 and IUF disappearance were determine by Chi-squared test. To determine the effect of predictor variables on open days, Cox proportional-hazards regression was used. The above mentioned variables were used as predictor variables. Censor criteria were cows that

were culled before confirmation of pregnancy or cows remaining open at the end of the study period (150 days on milk).

After this initial screening including all cows (affected by one or two criteria at the same time), two subsequent analyses were conducted on animals affected by only one inclusion criterion (presence of echogenic fluid or increased measures of the genital tract) to determine the effect of treatment on both groups of postpartum cows. These Cox proportional-hazards regressions were performed considering the same predictor variables as in previous analysis.

Binary logistic regression analysis was performed considering fetal loss following pregnancy diagnosis as the dependant variable and above mentioned variables as independent factors. Logistic regression analysis was performed according to the method of Hosmer and Lemeshow (Hosmer and Lemeshow 1989).

3. RESULTS

One hundred and forty dairy cows with abnormal uterine involution on Days 15-21 postpartum entered in this study. Lactation number, interval parturition-gestation and number of inseminations (mean \pm SD) were 2.19 ± 1.5 and 104 ± 30 days, and 2.39 ± 1.72 AI, respectively. At Day 150 postpartum, 124 cows (88.57%) had been diagnosed as pregnant and 20 of them (16.1%) suffered from early pregnancy loss.

One hundred and ten cows had echogenic IUF at V1. FIU content disappeared at V2 on 55 control and 35 treated cows (80.9% vs 87.5%, $p > 0.1$). Only 10 animals had persistent echogenic IUF at V3 (8 control and 2 treated cows, 11.4% vs 5%, $p > 0.1$) and were treated with a second dose of PG.

Based on the hazard ratio, the likelihood of pregnancy was lower in multiparous compared with primiparous cows and in cows with abnormal uterine findings on V3 compared with normal uterine findings on V3 (by factors of 0.56 and 0.35, respectively) (Figure 1,2). Cows with peripartum disorders tended to be less likely to become pregnant (by a factor of 0.57, $p = 0.052$) (Table 1).

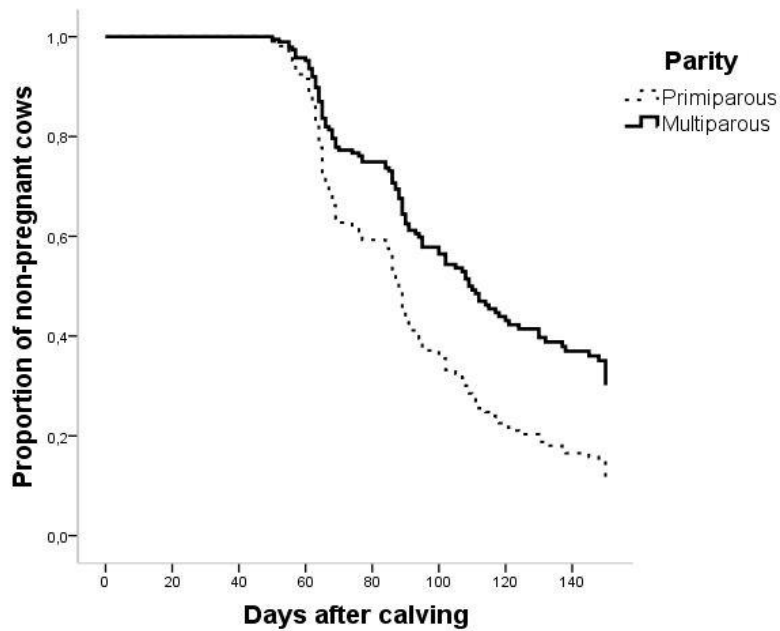


Figure1. Cox's proportional hazards regression survival curves for analysis of time of becoming pregnant until Day 150 postpartum for cows suffering endometritis (presence of echogenic intrauterine fluid, cervical diameter of ≥ 4 cm or endometrial thickness of ≥ 0.75 cm) on Days 15-21 postpartum, for multiparous (n=80) and primiparous (n=63) animals.

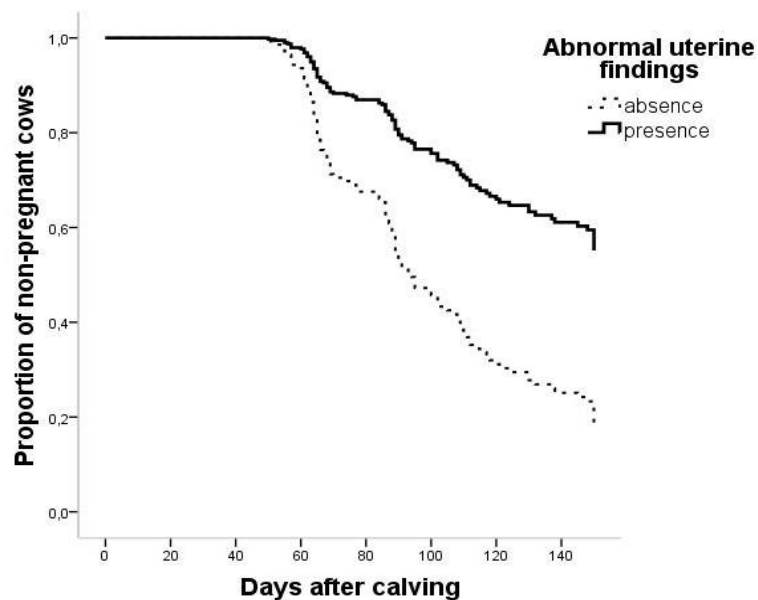


Figure2. Cox's proportional hazards regression survival curves for analysis of time of becoming pregnant until Day 150 postpartum for cows suffering endometritis (presence of echogenic intrauterine fluid, cervical diameter of ≥ 4 cm or endometrial thickness of ≥ 0.75 cm) on Days 15-21 postpartum, for cows with presence (n=10) and absence (n=130) of abnormal uterine involution on Days 25-39 postpartum.

Table 1 Final Cox's proportional hazards regression models of factors that were associated with time to conception in abnormal uterine postpartum cows (presence of echogenic intrauterine fluid, cervix ≥ 3.5 cm and/or endometrium thickness ≥ 0.75 cm on Days 15-21 postpartum).

Factor	Class	n	HR pregnancy	95% CI	P - value
Parity	Primiparous	60	Reference	-	-
	Multiparous	80	0.56	0.38-0.82	< 0.01
Abnormal uterine findings on V3*	Absence	130	Reference	-	-
	Presence	10	0.35	0.13-0.97	0.04
Peripartum disorders†	Absence	117	Reference	-	-
	Presence	23	0.57	0.31-1.005	0.052

P value for the model 0.001. CI, confidence interval for the Hazard Ratio. HR, Hazard ratio

* Cows suffering from presence of echogenic intrauterine fluid on Days 29-35 postpartum

† dystotic parturition, stillbirth, previous twin pregnancy and/or placenta retention.

A second Cox proportional-hazards regression was performed in cows suffering from presence of echogenic IUF without enlargement of measures of the genital tract at V1. Based on the hazard ratio, the likelihood of pregnancy was lower in multiparous compared with primiparous cows and in cows with abnormal uterine findings on V3 compared with normal uterine findings on V3 (by factors of 0.26 and 0.29, respectively). Cows with presence of CL were more likely to conceive than cows without CL (by a factor of 2.29) (Figure 3). Animals with previous peripartum disorders tended to be less likely to become pregnant ($p=0.08$) (Table 2).

Table2. Final Cox's proportional hazards regression models of factors that were associated with time to conception in postpartum cows suffering from presence of echogenic intrauterine fluid without enlargement of measures of the genital tract on Days 15-21 postpartum.

Factor	Class	n	HR pregnancy*	95% CI	P - value
Parity	Primiparous	22	Reference	-	-
	Multiparous	28	0.26	0.13-0.54	< 0.01
Abnormal uterine findings on V3*	Absence	44	Reference	-	-
	Presence	6	0.29	0.08-0.98	0.04
Peripartum disorders†	Absence	41	Reference	-	-
	Presence	9	0.424	0.16-1.11	0.08
Corpus luteum V1▪	Absence	28	Reference	-	-
	Presence	22	2.29	1.16-4.52	0.02

P value for the model 0.001. CI, confidence interval for the Hazard Ratio. HR, Hazard ratio

* Cows suffering from presence of echogenic intrauterine fluid on Days 29-35 postpartum

† dystotic parturition, stillbirth, previous twin pregnancy and/or placenta retention.

▪ Presence of corpus luteum on Days 15-21 postpartum

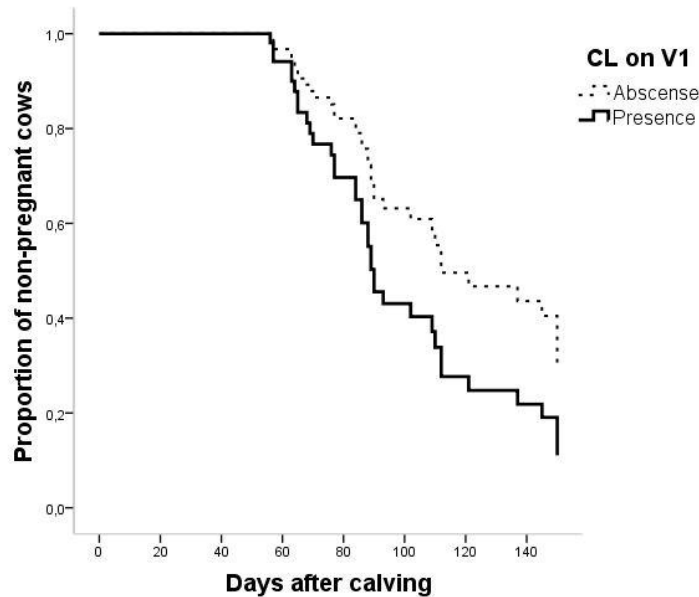


Figure3. Cox's proportional hazards regression survival curves for analysis of time of becoming pregnant until Day 150 postpartum for cows suffering from presence of echogenic intrauterine fluid without increased measures of the genital tract on Days 15-21 postpartum (V1), for cows with presence (n=22) and absence (n=28) of corpus luteum on Days 15-21 postpartum.

Finally, one last Cox proportional-hazards regression was performed in cows with increased measures of the genital tract without presence of echogenic IUF at V1. Based on the hazard ratio, multiparous cows tended to be less likely to conceive than primiparous cows ($p=0.055$).

The binary logistic regression analysis of early pregnancy losses showed no significant effects of any of the variables studied.

4. DISCUSSION

Our results indicated no effect of a single dose of PG on Days 15-21 postpartum of cows suffering endometritis on the subsequent conception rate or uterine involution, even in cyclic animals. Moreover, cows with IUF at V1 that early return to ovarian cyclicity after calving, had better reproductive performance than the remaining animals.

Luteolytic and uterotonic effect of prostaglandins are thought to benefit postpartum cow by enhancing uterine immunity (Roth *et al.* 1983; Lewis 2004; Singh *et al.* 2008) and promoting the contraction of the smooth muscles in the uterus (Kindahl *et*

al. 1992). However, field studies show different results, supporting (Bonnett *et al.* 1990; Galvão *et al.* 2009; Salasel and Mokhtari 2011) or disapproving (López-Gatius and Camón-Urgel 1989; Kaufmann *et al.* 2010; Dubuc *et al.* 2011) the use of PG 15-21 days after calving. In this study, the treatment was not related with the subsequent conception rate or abnormal uterine status on subsequent examinations. It is known that plasma PG level (as inferred from plasma 13,14-dihydro- ketoprostaglandin F₂α concentrations; PGFM) peaks at parturition and gradually declines until third week postpartum to basal concentrations (Edqvist *et al.* 1978; Lewis *et al.* 1984; Nakao *et al.* 1997). However, levels of PGFM are higher in cows with uterine infections compared with healthy postpartum cows on weeks 3 to 5 (Del Vecchio *et al.* 1994). It is possible that levels of PGFM are already elevated in cows suffering from endometritis. This fact would perfectly explain the ineffectiveness of exogenous PG treatment on Days 15-21 postpartum. Thus, the question that arises is whether PG treatment can be effective or not on subsequent postpartum days. Maybe PG will only be an appropriate therapeutical protocol for cows suffering endometritis when individual endogenous PG is already reestablished.

Cyclic cows at V1 did not response better to treatment compared to the remaining animals. However, resumption of ovarian cyclicity before 21 days postpartum improved subsequent conception rate compared to anestrous cows in cows suffering IUF, but not in others (animals with IUF and enlargement of cervix or endometrium). Progesterone, although nowadays is under discussion (Subandrio *et al.* 2000), seems to have immunosuppressive actions (Lewis 2004). Thus, PG is usually applied to postpartum animals to induce a new follicular wave (LeBlanc 2008). Cows suffering from both factors, IUF and enlargement of cervix or endometrium, are probably close from suffering severe endometritis. Thus, neither the immunostimulating effects of estradiol are capable of improve their uterine status. Results of this study demonstrate no benefit of shortening the luteal phase in cyclic cows, as previous reports (Stevenson and Call 1983; Galvão *et al.* 2010), but gives support to the idea of crosstalk between uterus and ovary during not only the postpartum period, but also the entire reproductive cycle.

Placenta retention, dystotic parturition, stillbirth or previous twin pregnancy were associated to an impairment of subsequent conception rate, in agreement with

other studies (Grön *et al.* 1990; Fourichon *et al.* 2000; Mee 2008; López-Gatius *et al.* 2006). These peripartum events are usually associated with human manipulation and genital wounds that exacerbate the postpartum bacterial infection. An inflammatory response caused by these postpartum events, may delays uterine involution, increasing genital tract measures or producing IUF (Azawi 2008) and, as a result, increasing days open of cows. Due to ineffectiveness of PG treatment on early postpartum period, interest should be focus on preventing peripartum events.

Finally, it is well knows that multiparous cows were less likely to become pregnant compared with primiparous cows (Yániz *et al.* 2006, García-Ispierto *et al.* 2007). The effect of previous parturitions may affect the genital tract and the body condition score of the animals.

In conclusion, no effect of treatment with PG on Days 15-21 postpartum was observed on subsequent reproductive performance of high producing dairy cows, even in cyclic animals. Irrespective of treatment, early resumption of ovarian cyclicity enhances postpartum recovery and improves subsequent conception rate.

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CHAPTER 3

IS INTRAUTERINE THERAPY OF ABNORMAL UTERINE INVOLUTION USEFUL IN HIGH PRODUCING DAIRY COWS?

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IS INTRAUTERINE THERAPY OF ABNORMAL UTERINE INVOLUTION USEFUL IN HIGH PRODUCING DAIRY COWS?

Abstract

The aims of this study were to evaluate by transrectal ultrasonography the effect on cows suffering endometritis on days 15-21 postpartum of a single intrauterine infusion of penicillin or a single large-volume lavage with physiologic saline solution. One hundred and one cows were examined on Days 15-21 (V1), 22-28 (V2) and 29-35 (V3) postpartum. Cranial cervical size, endometrial thickness and presence of intrauterine fluid (IUF) were recorded by transrectal ultrasonography. Cows considered as suffering endometritis included the following criteria: presence of echogenic IUF, cervical diameter of ≥ 4 cm or endometrial thickness of ≥ 0.75 cm. Cows without presence of corpus luteum were randomly assigned to a control (n= 40), penicillin (n=29) or saline group (n=32) and classified as presenting abnormal (presence of echogenic IUF or increased genital tract measures) or normal uterine findings in V3. Animals in the penicillin and saline group were treated with a single intrauterine lavage with 30 ml of penicillin or with a single intrauterine lavage with 200ml of physiological saline solution at V1. Chi-square test revealed no relationship between abnormal uterine findings in V3 and group of treatment in V1. Cox proportional-hazards regression determined that, based on the hazard ratio, multiparous and cows with calving in warm season were less likely to become pregnant by factors of 0.56 and 0.50 compared to the remaining cows, respectively. A second analysis performed in cows with abnormal involution revealed that the likelihood of return to ovarian cyclicity was 2.25 times higher in multiparous compared with primiparous cows. Moreover, animals calving during warm season tended to be more likely to return to ovarian cyclicity compared with calving in cool season (p=0.051). Through binary logistic regression procedures it was determined that cows receiving a previous treatment on first week after parturition were 2.96 times more likely to suffer fetal loss compared with non treated cows. In summary, no effect was observed of a single intrauterine infusion of penicillin or normal saline serum on Days 15-21 postpartum of cows suffering endometritis on the

subsequent conception rate or return to cyclicity. Moreover, calving season were intimately related to reproductive performance.

Keywords: transition period, bovine, genital tract, puerperium, metritis, fertility, ultrasound.

1. INTRODUCTION

Parturition is the most important step of the reproductive cycle of mammals. Complications in this process may affect the subsequent reproductive performance of dams. Domestic animals, and especially dairy cows, are adapted to the new high producing systems, in which postpartum recovery is even more complex, due to the postpartum immunosuppression of high milk production (Dobson *et al.* 2007; Walsh *et al.* 2011). Thus, factors such as management, heat stress and nutrition need to be carefully implemented in herds to minimize the negative effects of peripartum period on metabolic status and uterine health of the cows (Goff and Horst 1997; García-Ispuerto 2007a; Goff 2008).

Prepartum intrauterine environment is considered to be sterile but becomes contaminated during the first days after parturition with a wide range of bacteria (Földi *et al.* 2006). However, this contamination is not always associated with uterine infection. Uterine disease is a multifactorial process depending on factors such as, the species and virulence of bacteria and the contamination load of the uterine lumen (Sheldon *et al.* 2006; LeBlanc *et al.* 2011), and the immune status of the cow (Singh *et al.* 2008). Since postpartum immunity is depressed during 2-3 weeks after parturition (LeBlanc 2008) and neutrophil function is significantly impaired (Hammon *et al.* 2006), it is easy to understand that some dams develop uterine infection such as metritis or endometritis.

Therapy for uterine infection should eliminate pathogens from the uterus by evacuating uterine fluids or by exposing the bacteria to effective drugs (Azawi 2008), such as intrauterine antibiotics (Paisley *et al.* 1986; LeBlanc 2008). Apart from not creating new resistances, good penetration into the subendometrial layers, limited

systemic absorption, lack of irritation, and the maintenance of antibacterial activity in the environment of the uterus are the characteristics needed for an appropriate intrauterine antibiotic treatment (Paisley *et al.* 1986; Santos *et al.* 2010; Malinowski *et al.* 2011). For that reason, several intrauterine antibiotics have been tested, but there are still no conclusive results (Lewis 1997; LeBlanc 2008). Another therapeutic strategy could be large-volume lavage with physiologic saline solution, used in mares for evacuating intrauterine fluids and assisting bacteria elimination (Troedsson *et al.* 1995). However, to the best of our knowledge, no field trial has been performed on dairy cows for treating endometritis with saline solution lavages.

Therefore, the aims of our study were to evaluate by transrectal ultrasonography the effect on cows suffering from endometritis on days 15-21 postpartum of: (1) a routine postpartum practice established in a farm, based on a single intrauterine infusion of penicillin, and (2) a single large-volume lavage with physiologic saline solution.

2. MATERIAL AND METHODS

2.1. Cattle and herd management

The study was performed on a commercial herd comprising 650 high-producing Holstein-Friesian dairy cows in northeastern Spain. The study population included 140 clinically normal postpartum dairy cows that had calved from May 2010 to December 2011. Herd management involved the use of pedometers, housing in free stalls with concrete slatted floors and cubicles, daily postpartum checks and a weekly reproductive health program.

The mean annual culling rate and milk production during the study period were 28% and 10.650 kg / cow, respectively. The cows were grouped according to age (primiparous vs multiparous), milked three times daily and fed complete rations. Feeds consisted of cotton seed hulls, barley, corn, soybean, bran and roughage, primarily corn, barley or alfalfa silage and alfalfa hay. Rations were in line with NRC recommendations (NRC 2001). Dry cows were kept in a separate group and transferred, depending on their body condition score and age, 7–25 days prior to parturition to a ‘parturition group’. An early postpartum, or ‘fresh cows’, group was also established for postpartum

nutrition and control. Clinically normal cows were transferred to separate groups from 7 days postpartum onwards. Clinically normal animals are defined as cows free of diseases such as lameness, mastitis, digestive disorders and puerperal metritis.

2.2. Reproductive management

In postpartum daily checks, the following puerperal diseases were treated until resolved or until culling: signs of injury to the genital area (i.e. vaginal or recto-vulvar lacerations), metabolic diseases such as hypocalcaemia and ketosis (the latter, diagnosed during the first or second week postpartum), retained placenta (fetal membranes retained longer than 12 h after parturition) or primary metritis (diagnosed during the first or second week postpartum in cows not suffering placental retention). Cows with a retained placenta or primary metritis were always treated with oxytetracycline boluses introduced into the uterus.

The voluntary waiting period for the herd was 50 days in milk. Cows from 51 to 57 d postpartum with no uterine or ovarian disorders detected by ultrasound were fitted with a progesterone releasing intravaginal device (PRID, containing 1.55 g of progesterone, CEVA Salud Animal, Barcelona, Spain) left for 9 days. These animals were also given prostaglandin F_{2α} (25 mg dinoprost i.m.; Enzaprost, CEVA Santé Animale, Libourne, France) 24 h before PRID removal (García-Ispierto *et al.* 2010). Moreover, estrus was detected by pedometers and confirmed by palpation per rectum at AI (López-Gatius and Camon-Urgel 1991). All animals were artificially inseminated with frozen semen from bulls of proven fertility. If cows returned to estrus, their status was confirmed by ultrasound, and the animals were recorded as non-pregnant. In the remaining cows, pregnancy diagnosis was performed by ultrasound 28–34 days post-insemination. Fetal loss was recorded when the 90–96 days-diagnosis proved negative.

2.3. Clinical examination

During the weekly herd reproductive visit, a transrectal ultrasonography exam was performed on Days 15-21 (V1), 22-28 (V2) and 29-35 (V3) postpartum by the same veterinarian. The entire reproductive tract was examined by ultrasound using a portable B-mode ultrasound scanner (Easi-scan with a 7.5 MHz transducer). Scanning was

performed along the dorsal/lateral surface of each horn and cervix. Cranial cervical size and endometrial thickness were measured using the internal calipers of the ultrasonograph, and intrauterine fluid (IUF) was scored as absent, anechogenic or echogenic as previously reported (López-Helguera *et al.* 2012). Ovarian structures in both ovaries were also recorded. After V3, three weekly consecutive ultrasonography exams were performed to determine ovarian structures. When a corpus luteum was detected, cow was considered to be returned to ovarian cyclicity.

4.4. Treatment

Based on previous experimental findings (López-Helguera *et al.* 2010, 2012), cows considered as suffering endometritis included the following criteria: presence of echogenic IUF, cervical diameter of ≥ 4 cm or endometrial thickness of ≥ 0.75 cm. Cows receiving reproductive hormone administration, systemic antibiotic or intrauterine therapy in the current lactation within seven days prior to V1, were excluded from the study. Cows suffering endometritis without presence of corpus luteum, were randomly assigned into a control (n= 40), penicillin (n=29) and saline group (n=32). Animals in the penicillin group were treated with a single intrauterine lavage with 30 ml of penicillin (300mg/ml benzilpenicilin procain; Depocillin, Intervet SA, Aprilia, Italy) in V1. After antibiotic administration, a uterus massage was performed for a good distribution of drug along uterine horns. Cows in saline group were treated with a single intrauterine lavage with 200ml of physiological saline solution (NaCl 0.9% solution; B.Braun) in V1. After saline serum administration, a uterus massage was performed for stimulating the evacuation of intrauterine content. Animals assigned to control group received no treatment. Cows were reexamined on the following weeks by ultrasonography. In case of presence of echogenic IUF on V1, IUF disappearance or permanence was evaluated in V2. When echogenic IUF persisted or abnormal uterine findings were observed on V3, a single dose of prostaglandin (PG) treatment was administrated (25 mg dinoprost i.m.; Enzaprost, CEVA Santé Animale, Libourne, France).

2.5. Data Collection and analysis

The variables recorded for each cow were: parity (primiparous vs multiparous), season of parturition (warm –May to September- vs cool -October to April) (Labèrnia *et al.* 1998; López-Gatius 2003), type of parturition (normal vs dystotic), stillbirth, previous twin pregnancy, retention of the placenta (retention of the fetal membranes >12 hours), antibiotic treatment on first week after calving (presence or absence), treatment group (0=control vs 1=penicillin vs 2=saline), abnormal uterine findings on V3 (presence or absence of echogenic IUF), date of return to cyclicity, date of conception, days open, presence of twins and early pregnancy loss (<90 days of pregnancy).

Previous disorders such as dystotic parturition, stillbirth, previous twin pregnancy and placenta retention, were pooled into one single category (peripartum disorders) and coded as a dichotomous variable where 1 denotes presence and 0 denotes absence. Parity, season of parturition, treatment group, previous treatment, abnormal uterine findings on V3, IUF disappearance, presence of twins and fetal loss following pregnancy diagnosis were also coded as dichotomous variables.

All statistics procedures were performed using the SPSS package version 18.0 (SPSS 20 Inc., Chicago, IL, USA) with the level of significance set at $P < 0.05$. Associations between treatment group, abnormal uterine findings on V3 and IUF disappearance were determined by Chi-squared test. To determine the effect of predictor variables on open days and on days to return to cyclicity, Cox proportional-hazards regressions were used. The above mentioned variables were used as predictor variables. Censor criteria were cows that were culled before confirmation of pregnancy or cows remaining open at the end of the study period (150 days on milk).

Binary logistic regression analysis was performed considering fetal loss following pregnancy diagnosis as the dependant variable and above mentioned variables as independent factors. Logistic regression analysis was performed according to the method of Hosmer and Lemeshow (Hosmer and Lemeshow 1989).

3. RESULTS

One hundred and one acyclic dairy cows with abnormal uterine involution on Days 15-21 postpartum entered in this study. Lactation number, interval parturition-return to cyclicity, parturition-gestation and number of inseminations (mean \pm SD) were 2.6 ± 1.7 , 39.3 ± 11.8 , 103.7 ± 35 days and 2.5 ± 1.7 AI, respectively. At Day 150 postpartum, 72 cows (71.3%) had been diagnosed as pregnant. At the end of the study, 79 cows became pregnant and 19 of them (24.1%) suffered from early pregnancy loss.

Ninety four cows had echogenic IUF at V1. IUF disappeared at V2 on 25 control, 27 penicillin and 23 saline treated cows (75.7% vs 84.4% vs 79.3%, $p > 0.1$). Only 13 animals had persistent echogenic IUF at V3 (3 control, 5 penicillin and 2 saline treated cows, 9.1% vs 15.6% vs 17.2%, $p > 0.1$) and were treated with a single dose of PG.

Based on the hazard ratio, the likelihood of pregnancy was lower in multiparous compared with primiparous cows and in cows with calving in warm season compared with calving in cool season (by factors of 0.56 and 0.50, respectively) (Table1) (Figure1).

Table 1 Final Cox's proportional hazards regression models of factors that were associated with time to conception in abnormal uterine postpartum cows (presence of echogenic intrauterine fluid, cervix ≥ 3.5 cm and/or endometrium thickness ≥ 0.75 cm on Days 15-21 postpartum).

Factor	Class	N	HR pregnancy	95% CI	P - value
Parity	Primiparous	37	Reference	-	-
	Multiparous	64	0.562	0.34-0.92	0.02
Season of calving *	Cool	63	Reference	-	-
	Warm	38	0.507	0.31-0.81	< 0.01

P value for the model 0.001. CI, confidence interval for the Hazard Ratio. HR, Hazard ratio

* Cool= October to April; Warm= May to September.

Table 2 shows the hazard ratios of the variables included in the second Cox proportional-hazards regression. Based on the hazard ratio, the likelihood of return to ovarian cyclicity was 2.25 times higher in multiparous compared with primiparous

cows. Animals calving during warm season tended to be more likely to return to ovarian cyclicity compared with calving in cool season (p=0.051).

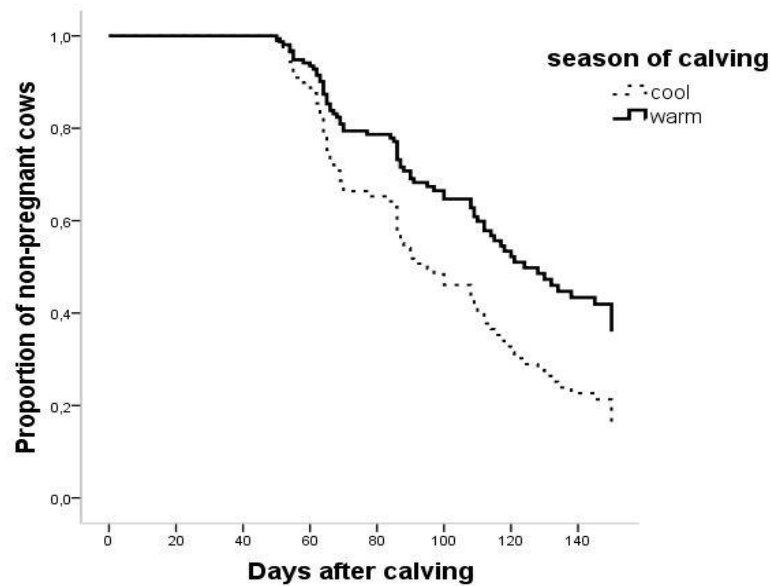


Figure 1. Cox's proportional hazards regression survival curves for analysis of time of becoming pregnant until Day 150 postpartum for cows suffering endometritis (presence of echogenic intrauterine fluid, cervical diameter of ≥ 4 cm or endometrial thickness of ≥ 0.75 cm) on Days 15-21 postpartum, for cows with calving in warm (n=38) and cool (n=63) season.

Table 2 Final Cox's proportional hazards regression models of factors that were associated with time to return to ovarian cyclicity in abnormal uterine postpartum cows (presence of echogenic intrauterine fluid, cervix ≥ 3.5 cm and/or endometrium thickness ≥ 0.75 cm on Days 15-21 postpartum).

Factor	Class	n	HR pregnancy	95% CI	P - value
Parity	Primiparous	37	Reference	-	-
	Multiparous	64	2.25	1.35-3.76	< 0.01
Season of calving *	Cool	63	Reference	-	-
	Warm	38	1.58	0.99-2.54	0.051

P value for the model 0.001. CI, confidence interval for the Hazard Ratio. HR, Hazard ratio

* Cool= October to April; Warm= May to September.

Finally, the binary logistic regression analysis showed a significant effect of previous treatment on early pregnancy losses. Based on the odds ratio, cows receiving a previous treatment on first week after parturition were 2.96 times more likely to suffer fetal loss (n=12/34, 35%) compared with non treated cows.

4. DISCUSSION

Our results showed no effect of a single intrauterine infusion of penicillin or physiologic saline serum on Days 15-21 postpartum of cows suffering endometritis on the subsequent conception rate or resumption of ovarian cyclicity. Surprisingly, despite cows calving during warm season get pregnant later, returned earlier to cyclicity compared to the remaining cows.

Bacterial contamination of uterus has to be carefully understood to implement a local uterine therapy. Recent studies show that *Escherichia coli* is frequently isolated from uterus in the first week postpartum and is associated with incidence of metritis and with an increased risk of infection with *Arcanobacterium pyogenes* and *Fusobacterium necrophorum* in weeks 2 and 3 postpartum (Dohmen *et al.* 2000; Azawi 2008). These two bacteria has been related to clinical endometritis (William *et al.* 2005; Bicalho *et al.* 2012) and seems to be synergetic, causing severe cases of infection in some animals (Ruder *et al.* 1981). It has been demonstrated that bacterial etiology of postpartum uterine disease is dynamic and multifactorial (Bicalho *et al.* 2012). Thus, not only these two pathogens will be involved in endometritis (Paisley *et al.* 1986; Williams *et al.* 1988; Frazier 2001; Donofrio *et al.* 2009). The key in any bacterial contamination therapy is antibiotic sensibility and resistance. Penicillin administrated by intrauterine infusion had been used in routine practice for uterine disease (Thurmond *et al.* 1993). In this study, intrauterine infusion of penicillin did not improve conception rate or resumption of ovarian cyclicity of cows with endometritis. Although *F. necrophorum* (Gramm negative bacteria) has an elevated sensibility to penicillin, *A. pyogenes* has been recently described to reach from 31.3% (Malinowski *et al.* 2011) to 86.1% (Santos *et al.* 2010) of penicillin resistance. Moreover, some of these strains can produce penicillinases that would interfere on effectiveness of intrauterine treatment (Hussain and Daniel 1991; Lewis 1997). For all that reasons, penicillin should not be used for intrauterine therapy in cow suffering endometritis.

Saline serum lavage did not improve either conception rate or return to ovarian cyclicity of cows with endometritis. The presence of pathogenic bacteria in the uterus causes inflammation and histological lesions of the endometrium (Bonnett *et al.* 1991) and delays uterine involution (Sheldon *et al.* 2006). This therapy should eliminate

pathogens from the uterus by evacuating uterine fluids and reduce its impact on reproductive performance (Azawi 2008). However, in this study negative effects of bacterial infection are not significantly diminished. Probably, the presence of pus damage endometrial tissue and although the content is evacuated, the animal needs extra-uterine support to overcome the infection.

Our results demonstrated a negative effect of treatment during first week postpartum on subsequent early pregnancy loss. Animals treated during first week after parturition were affected by puerperal metritis, generally related to *E. coli* contamination (Huszenicza *et al.* 1999). This bacterial infection not only delays uterine involution, but also disturbs embryo survival (Semambo *et al.* 1991). *E. coli* generates endotoxins that disrupt the endometrial integrity allowing its absorption to systemic circulation (Dohmen *et al.* 2000). As a result, systemic endotoxins increase and there is a PG release, shortening the lifespan of corpus luteum (Peter and Bosu 1987; Gilbert *et al.* 1990). Moreover, endotoxemia has been also suggested to induce fetal death (Gilbert *et al.* 1990). Thus, intrauterine infection can endanger fetus viability, impairing reproductive performance.

Heat stress has been widely documented to reduce reproductive performance in dairy cows (De Rensis and Scaramuzzi 2003; García-Ispierto *et al.* 2007a). The results of this study showed a decreased conception rate on cows with parturition during warm compared to cool season. Lower concentration of plasma estradiol and a possible decrease on plasma progesterone on warm period could be affecting follicular development, embryonic implantation and its viability (De Rensis and Scaramuzzi 2003). Despite of this well known effect of heat stress, in this study cows calving during warm period, tended to be more likely to return to ovarian cyclicity. According to Opsomer *et al.* (2000), nutritional changes in pasture would explain this improvement, but not in intensive high producing dairy cattle. Although cattle is not strictly seasonal breeder, it is well known that photoperiod affect its reproductive performance (Hansen and Hauser 1984). Thus, photoperiod has been included in statistical model for conception rate and return to ovarian cyclicity (data not shown). No significant effect has been found. Thus, heat stress has an effect *per se* in ovarian cyclicity in this study. The question that arises here is which mechanism heat stress is using to reduce the interval calving-first ovulation. It is possible that follicular growth and luteolytic

mechanisms were compromised in heat stressed cows (Wilson *et al.* 1998). Although they return to cyclicity before the remaining animals, low plasma progesterone concentration may impair subsequent reproductive functions and cause low fertility (Ronchi *et al.* 2001).

Finally, an effect of parity on conception rate and resumption of ovarian cyclicity was observed. It is well known that multiparous cows had an impaired fertility compared to primiparous (López-Gatius *et al.* 2006; García-Ispuerto *et al.* 2007b), that seems to be associated with the increased incidence of peripartum disorders (Gröhn *et al.* 1990). At the same time, an earlier return to ovarian cyclicity has been related to multiparous cows (Tanaka *et al.* 2008; Santos *et al.* 2009), supporting the results of this study.

In summary, no effect was observed of a single intrauterine infusion of penicillin or normal saline serum on Days 15-21 postpartum of cows suffering endometritis on the subsequent conception rate or return to cyclicity. Moreover, calving season were intimately related to reproductive performance.

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CHAPTER 4

REPRODUCTIVE PERFORMANCE OF ANOESTROUS HIGH-PRODUCING DAIRY COWS IMPROVED BY ADDING EQUINE CHORIONIC GONADOTROPHIN TO A PROGESTERONE-BASED OESTRUS SYNCHRONIZING PROTOCOL

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REPRODUCTIVE PERFORMANCE OF ANOESTROUS HIGH-PRODUCING
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GONADOTROPHIN TO A PROGESTERONE-BASED OESTRUS
SYNCHRONIZING PROTOCOL

Abstract

This study sought to improve the reproductive performance of anoestrous high-producing dairy cows by including equine chorionic gonadotrophin (eCG) after progesterone-releasing intravaginal device (PRID) removal. In Experiment I, 806 cows at 51 to 57 d postpartum were randomly assigned to a PRID (treated with PRID), PRID-500 (treated with PRID plus 500 IU of eCG) or PRID-750 (treated with PRID plus 750 IU of eCG) group. In Experiment II, 422 cows showing a long anoestrus period (animals with no oestrus signs nor luteal tissue 35 d before treatment), were randomly assigned to the PRID, PRID-500 or PRID-750 groups. The dependent variables considered in binary logistic regression analyses for both experiments were the rates of oestrus, ovulation and conception after treatment, the cumulative conception rate on Day 120 postpartum and pregnancy loss. In Experiment I, interaction between treatment and season showed a significant effect on the oestrus response. Thus, during the warm season, PRID group cows were 8.9 times more likely to express oestrus than the remaining cows. Moreover, inseminated cows with two or more corpora lutea 8-14 d after treatment were more likely to become pregnant (by a factor of 2.4) than cows with a single corpus luteum. Finally, cows without luteal structures treated with PRID were 0.4 less likely to be pregnant on Day 120 postpartum, compared to the remaining cows. In Experiment II, cows in the PRID group treated during the warm or cool season were less likely to exhibit oestrus (by a factor of 0.06 or 0.2, respectively) or ovulate (by a factor of 0.004 or 0.14, respectively) than the remaining cows. In conclusion, in anoestrous cows in both experiments, the addition of eCG to the use of an intravaginal progesterone device to induce oestrus was beneficial. The recommended dose of eCG is 500 IU.

Keywords: double ovulation, oestrus synchronization, eCG, dairy cattle, progestagens

1. INTRODUCTION

Over the past few decades, fertility has been constantly declining in dairy cows (Lucy 2001; López-Gatius 2003). Reasons for subfertility are multifactorial and cannot be entirely linked to increased milk production (López-Gatius et al. 2006; Garcia-Ispierto et al. 2007). Besides, recent evidence suggests that postpartum anoestrus is today the most important factor determining herd economy (Yániz et al. 2008). The postpartum period has therefore been identified as critical for the reproductive performance of a cow, when animals should return to normal ovarian cyclicity and become pregnant again (Morrow et al. 1969).

Higher producers show elevated clearance of steroid hormones from the circulation, leading to lower progesterone levels compared to heifers (Sartori et al. 2002; Wolfenson et al. 2004). Thus, it could be that exogenous progesterone during the postpartum may raise systemic progesterone levels despite the increased hepatic metabolism of high producing dairy cows. Progesterone-based treatments involving the use of a progesterone-releasing intravaginal device (PRID) have significantly improved the synchronization of oestrus and likelihood of pregnancy after first insemination in anovulatory cows (Larson and Ball 1992; Nebel and Jobst 1998; López-Gatius et al. 2001; López-Gatius et al. 2004; López-Gatius et al. 2008; Garcia-Ispierto et al. 2010), although many other measures are being currently used to try to improve reproductive efficiency.

In most mammals, reproductive success is determined by the physiological process whereby gonadotrophins (follicle stimulating hormone-FSH-, and luteinizing hormone-LH) act through specific receptors on the ovary to stimulate folliculogenesis and ovulation (Driancourt 2001). Hence, the administration of these hormones during both long anestrus and postpartum cows (Rostami et al, 2011), should help them return to ovarian cyclicity. Equine chorionic gonadotrophin (eCG) displays both FSH and LH-like activity and has a long half-life (Bever et al. 1989). The use of eCG at the time of PRID removal has been suggested to increase pregnancy rates in timed AI (TAI) programmes for suckled cows with a high prevalence of anoestrus (Baruselli et al. 2003;

Cutaia et al. 2003; Sá Filho et al. 2011a, b). However, to the best of our knowledge no investigation has examined the oestrus response to this strategy and subsequent reproductive performance in high-producing dairy cattle without using estradiol. The present study was designed to determine whether the placement of a PRID followed by eCG treatment given on the day of device removal in cows at 51-57 days postpartum or in anoestrus period, after 120 days postpartum increases reproductive performance high producing dairy cows.

2. MATERIAL AND METHODS

2.1. Cattle and herd management

The data examined were obtained from a reproductive control programme conducted at the University of Lleida on three well managed, high-producing Holstein-Friesian dairy herds in northeastern Spain. The data were derived from 1236 mature cows over the period November 2008 to March 2011. Briefly, herd management included the following common features: the use of pedometers, housing in free stalls with concrete slatted floors and cubicles, the use of fans and water sprinklers in the warm season, rigorous postpartum checks, the same reproductive health programme, confirmation of oestrus at artificial insemination (AI) by palpation per rectum, and most AI (over 90%) performed by veterinarians.

The mean annual culling rate was 30%. Mean annual milk production for the herds over the study period was 10,590 kg. The cows were grouped according to age (primiparous versus multiparous), milked three times daily and fed complete rations. Feeds consisted of cotton seed hulls, barley, corn, soybean, and bran, and roughage, primarily corn, barley or alfalfa silage and alfalfa hay. Rations were in line with NRC recommendations (NRC 2001). Dry cows were kept in a separate group and transferred, depending on their body condition score and age, 7–25 d prior to parturition to a “parturition group”. An early postpartum, or “fresh cows”, group was established for postpartum nutrition and controls, and 7–20 d postpartum primiparous and multiparous lactating cows were transferred to separate groups. All cows were artificially inseminated. The voluntary waiting period for the herds was 50 d.

2.2. Reproductive health management

In postpartum checks (daily), the following puerperal diseases were treated until resolved or until culling: signs of injury to the genital area (i.e., vaginal or recto-vulvar lacerations), metabolic diseases such as hypocalcaemia and ketosis (the latter, diagnosed during the first or second week postpartum), retained placenta (foetal membranes retained longer than 12 h after parturition), or puerperal metritis (diagnosed during the first or second week postpartum in cows not suffering placental retention). The herds were maintained on a weekly reproductive health programme. This involved examining the reproductive tract of each animal by ultrasound (Easi-scan with a 7.5 MHz transducer) from 30 to 36 d postpartum to check for normal uterine involution and ovarian structures. Reproductive disorders diagnosed at this time such as endometritis or ovarian cysts were treated until resolved. Detectable intrauterine cloudy fluid was interpreted as endometritis. An ovarian cyst was diagnosed when a follicular structure larger than 20 mm in diameter (external diameter including the wall) was detected in either or both ovaries in the absence of a corpus luteum and uterine tone. Cows with a retained placenta or puerperal metritis were always treated with oxytetracycline boluses and prostaglandin $F_{2\alpha}$ at the end of treatment.

Cows with no oestrus signs before Days 71-77 in milk (DIM) were entered in a weekly reproductive programme and inseminated either following specific treatment depending on their ovarian structures (López-Gatius et al. 2008) or during natural oestrus. Briefly, ovarian structures and uterine status or contents were recorded. If a cow had a corpus luteum estimated to be at least 15 mm in diameter determined by ultrasonography the animal was treated with cloprostenol. If a cow was diagnosed as having an ovarian cyst, treatment was the same as described above for the postpartum period. A cow was considered to suffer follicular anovulation when a follicular structure of at least 8–15 mm was detected in two consecutive examinations in the absence of a corpus luteum or cyst, and no estrus signs were noted during the 7-day period between the exams (López-Gatius et al, 2004). A follicular structure of this size at least was always detected in the cows lacking cysts or luteal structures in the two consecutive exams. These cows were fitted with a progesterone releasing intravaginal device (PRID, containing 1.55 g of progesterone; CEVA Salud Animal, Barcelona, Spain). The PRID

was left in the animal for 9 days and these animals were also given 500 µg cloprostenol i.m. 8 days after insertion (López-Gatius et al, 2006).

2.3. Experiments

2.3.1 Experiment I. Postpartum cows

Postpartum Day 51 to 57 cows with no uterine or ovarian disorders detected by ultrasound were randomly assigned to one of the groups: PRID (treated with PRID, n=269), PRID-500 (treated with PRID plus 500 IU of eCG, n=269) or PRID-750 (treated with PRID plus 750 IU of eCG, n=268). Cows in the PRID group were fitted with a progesterone releasing intravaginal device (1.55 g progesterone; PRID, CEVA Salud Animal, Barcelona, Spain). The PRID was left for 9 d and these animals were also given prostaglandin F_{2α} (25 mg dinoprost i.m.; Enzaprost, CEVA Santé Animale, Libourne, France) 24 h before PRID removal (López-Gatius et al. 2008; Garcia-Ispuerto et al. 2010). Cows in the PRID-500 and PRID-750 groups received the same treatment plus eCG (500 IU or 750 IU, respectively; Syncostim, CEVA Santé Animale, Libourne, France) given on the day of PRID removal. Only healthy cows (with no signs of mastitis, lameness or digestive disorders) were included in the study. Ovarian structures were recorded the day of treatment by using ultrasonography. Corpora lutea were defined as small if they were smaller than 15 mm (mean of the maximum and minimum diameters), or normal if equal to or larger than 15 mm.

2.3.2. Experiment II. Cows showing long anoestrus

Cows showing more than 120 DIM and with no oestrus signs nor luteal tissue detected throughout 35 d before treatment were randomly assigned to the PRID (treated with PRID n=142), PRID-500 (treated with PRID plus 500 U of eCG n=144) or PRID-750 (treated with PRID plus 750 U of eCG n=136) groups. These cows had been included previously in the experiment I and failed to both estrous detection and luteal tissue formation. Cows in the different groups were treated as described in Experiment I.

2.4. Insemination and pregnancy diagnosis

Only cows showing oestrus signs were inseminated. Oestrus was detected using a pedometer system (AfiFarm System; SAE Afikim). Walking activity values were recorded at the milking parlour (three times daily) and analyzed automatically using the herd management computer programme. A walking activity greater than 80% of the mean activity recorded in the previous 2 d was taken as the lower limit for a cow to be considered in oestrus. Since these herds have been observed to show a very significant relationship between increased walking activity and fertility provided this increase is 80% to 993% (López-Gatius et al. 2005), values lower than 80% were not considered oestrus signs. Also taken into account was previous individual information concerning oestrus detection. For example, if a cow showed a 120% increase in activity yet during its two last oestrus periods the increase noted had been around 400%, the cow was not inseminated. Cows that exhibited oestrus within a 12-d interval were also discarded and registered as cows with possible reproductive disorders for inclusion in the weekly gynaecological exam programme. The cows were finally inseminated after oestrus had been confirmed by examination of the genital tract and vaginal fluid (López-Gatius and Camón-Urgel 1991; López-Gatius 2000). If cows returned to oestrus, their status was confirmed by examination per rectum, and the animals were recorded as non-pregnant. In the remaining cows, pregnancy diagnosis was performed by ultrasound 28–34 d postinsemination. Cows diagnosed as not pregnant were included in the weekly reproductive programme. Pregnancy was confirmed by palpation per rectum 90–96 d postinsemination. Foetal loss was recorded when the 90–96 d-diagnosis proved negative. Data from cows suffering any clinical disease before Day 120 DIM (open cows) or before Day 90 of gestation (pregnant cows) were withdrawn from the study. All gynaecological exams and pregnancy diagnoses were performed by the same veterinarian.

2.5. Data collection and analysis

2.5.1. Experiment I

The following data were recorded for each animal upon treatment: herd, number of oestruses, parity (primiparous versus multiparous), ovarian structures at treatment

determined by ultrasonography (follicle, small or mature corpus luteum), treatment (0 = PRID, 1= PRID-500, 2= PRID-750), season of treatment (cool - October to April - versus warm – May to September), oestrus detection rate within 20 d of treatment onset (before Days 71-77 DIM-ODR), milk production at treatment (mean production during the three days before treatment), AI date, semen providing bull, AI technician, ovulation (presence of a corpus luteum) 12 days after PRID removal (OVUL12), pregnancy diagnosis 28–34 d either subsequent to treatment (PRATE28-34) or following inseminations performed before Day 120 DIM (PRATE120) and foetal loss following a positive pregnancy diagnosis (PLOSS). The factors parity and season of treatment were coded as dichotomous variables, where 1 denotes presence (multiparous cows and the warm period, respectively) and 0 denotes absence. Herd, treatment, ovarian structures at treatment, semen providing bull, AI technician and ovulation, were considered as class variables. Milk production and number of oestruses before treatment were considered continuous variables.

Five binary logistic regression analyses were performed. The dependent variables considered in these five analyses, respectively, were ODR, OVUL12, PRATE28-34, PLOSS, and PRATE120.

2.5.2. Experiment II

The following data were recorded for each animal: herd, parity (primiparous versus multiparous), treatment (0 = PRID, 1= PRID-500, 2= PRID-750), season of treatment (cool versus warm), DIM and milk production at treatment (mean production for the three previous days), ODR, AI date, semen providing bull, AI technician, ovulation OVUL12, PRATE28-34 and PLOSS. The factors parity and season of treatment were coded as dichotomous variables, where 1 denotes presence and 0 denotes absence. Herd, treatment, semen providing bull and AI technician, were considered as class variables. Milk production and DIM at treatment were considered continuous variables.

Four binary logistic regression analyses were performed. The dependent variables considered in these four analyses, respectively, were ODR, OVUL12, PRATE28-34, and PLOSS.

Regression analyses were conducted according to the method of Hosmer and Lemeshow (Hosmer and Lemeshow 1989) by the logistic procedure of the SPSS package. Basically, this method involves five steps as follows: preliminary screening of all variables for univariate associations; construction of a full model using all the variables found to be significant in the univariate analysis; stepwise removal of non-significant variables from the full model and comparison of the reduced model with the previous model for model fit and confounding; evaluation of plausible two-way interactions among variables and assessment of model fit using Hosmer–Lemeshow statistics. Variables with univariate associations showing a $P < 0.25$ were included in the initial model. Modelling was continued until all the main effects or interaction terms were significant according to the Wald statistic at $P < 0.05$. Probabilities of $P < 0.05$ were considered significant.

3. RESULTS

In both experiments, all inseminations were performed within 2-5 days following PRID removal. Due to the fact that 500 UI and 750 UI of eCG were not different for any of the statistical analyses performed (data not shown), information presented below regrouped both eCG groups.

3.1. Experiment I

The first binary logistic regression analysis identified significant effects of lactation number and number of oestruses on the ODR. Based on the odds ratios, the likelihood of an ODR following treatment was: lower by a factor of 0.9 in cows with a higher lactation number and higher by a factor of 1.45 in cows expressing prior oestrus for each unit increase in the number of lactations and oestruses respectively. The interaction between treatment and season was also found to be significant such that PRID group cows treated during the warm season were 8.9 times more likely to express oestrus than the remaining cows (Table 1).

Table 2 shows the odds ratios of the variables included in the second binary logistic regression model (PRATE28-34). The likelihood of conception after treatment was higher by a factor of 1.6 in cows expressing prior oestrus for each unit increase in

the number of oestruses. Inseminated cows with two or more corpora lutea 8-14 days after treatment were more likely to become pregnant (by a factor of 2.4) compared to cows with a single corpus luteum.

Table 1. Odds ratios of the variables included in the final logistic regression model for factors affecting ODR following treatment in postpartum cows.

Factor	Class	n	% Oestrous rate	Odds ratio	95% CI	P
Treatment x season	0 x cool	139/181	76.8	Reference		
	0 x warm	85/88	96.6	8.9	2.7-29.0	<0.001
	1 x cool	273/331	82.4	1.1	0.6-2.2	0.3
	1 x warm	170/205	82.9	1.6	0.7-2.9	0.16
Lactation number	Continuous	667/805	82.8	0.9	0.78-0.99	0.04
Prior oestruses	Continuous	667/805	82.8	1.45	1.04-2.08	0.02

P=0.0001 Hosmer and Lemeshow Goodness-of-fit test = 21.6; 3 df, P = 0.87.

Treatment: 0: PRID; 1: PRID + 500 IU or 750 IU eCG

CI:Confidence Interval

Table 2. Odds ratios of the variables included in the final logistic regression model for factors affecting PRATE28-34 after treatment in postpartum cows.

Factor	Class	n	%Conception Rate	Odds ratio	95% CI	P
Prior oestruses	Continuous	215/271	32.0	1.6	1.2-2.0	0.001
OS 12 days after treatment	Follicle	0/9	0	0.11	0.4-2.5	0.5
	CL	153/534	28.7	Reference		
	Two or more CL	62/128	48.4	2.4	1.4-3.4	<0.001

P=0.0001 Hosmer and Lemeshow Goodness-of-fit test = 21.6; 3 df, P = 1

CL: corpus luteum; OS: Ovarian structures; CI:Confidence Interval

The third binary logistic regression revealed significant effects of season and the oestrus rate on the PRATE120. Based on the odds ratios, the likelihood of being pregnant on Day 120 postpartum was lower by a factor of 0.58 in the warm compared to the cool season of treatment, and higher by a factor of 1.5 in cows expressing prior oestrus for each unit increase in the number of oestruses. The interaction treatment by ovarian structures at treatment was also found to be significant. This meant that PRID group cows without luteal structures at the beginning of treatment were 0.4 less likely to be pregnant on Day 120 postpartum, compared to the remaining cows (Table 3).

Table 3. Odds ratios of the variables included in the final logistic regression model for factors affecting PRATE120 in postpartum cows.

Factor	Class	n	%Conception rate	Odds ratio	95% CI	P
Season	Cool	336/530	65.5	Reference		
	Warm	152/292	51.5	0.58	0.4-0.8	0.01
Oestrous rate	Continuous	488/805	60.6	1.5	1.15-2.0	<0.001
No CL* x treatment	No CL x 0	29/81	35.8	0.4	0.2-0.6	0.04
	No CL x 1	109/184	59.23	0.8	0.5-2.7	0.5
	Other cows x all treatments	350/541	64.7	Reference		

P=0.0001 Hosmer and Lemeshow Goodness-of-fit test = 21.6; 3 df, P = 0.76.

*Cows with no luteal structures at the time of treatment

Treatment: 0: PRID; 1: PRID + 500 IU or 750 IU eCG ; CI:Confidence Interval

Finally, no significant effects of the variables examined were found on the OVUL12 and PL rate in cows becoming pregnant within 20 days of starting treatment.

3.2.Experiment II

Table 4 shows the odds ratios of the variables and interactions included in the first binary logistic regression (ODR). Cows in the PRID group, treated both during the warm and cool season, were less likely to express oestrus following treatment (by factors of 0.09 and 0.20 respectively) than the remaining cows.

Table 4. Odds ratios of the variables included in the final logistic regression model for factors affecting ODR following treatment in cows showing a long anoestrous period.

Factor	Class	n	% Oestrous rate	Odds Ratio	95% CI	P
Treatment x season	0-cool	27/71	38.0	0.20	0.10-0.5	<0.001
	0-warm	12/61	19.7	0.06	0.03-0.13	<0.001
	1-cool	103/132	78.0	0.9	0.34-1.7	0.9
	1-warm	123/160	76.9	Reference		

P=0.0001 Hosmer and Lemeshow Goodness-of-fit test = 21.7; 2 df, P = 0.9.

Treatment: 0: PRID; 1: PRID + 500 IU or 750 IU eCG

CI:Confidence Interval

Based on the odds ratio of the second binary logistic regression, the likelihood of cows OVUL12 decreased by a factor of 0.36 in herd 2 compared to herd 1. Interaction

between treatment and season was also found to be significant. Cows in the PRID group treated in the warm or cool season were 0.004 or 0.14 times, respectively, less likely to ovulate following treatment than the remaining cows (Table 5).

Table 5. Odds ratios of the variables included in the final logistic regression model for factors affecting OVUL12 following treatment in cows showing a long anoestrous period.

Factor	Class	N	% Ovulation rate	Odds Ratio	95% CI	P
Treatment x season	0-cool	42/71	59.2	0.14	0.05-0.28	<0.001
	0-warm	20/61	32.8	0.004	0.01-0.10	<0.001
	1-cool	125/139	89.9	0.69	0.4-1.4	0.5
	1-warm	139/160	87.0	Reference		
Herd	1	270/342	78.9	Reference		
	2	57/89	64.0	0.36	0.2-0.7	0.02

P=0.0001 Hosmer and Lemeshow Goodness-of-fit test = 21.6; 3 df, P = 0.87.

Treatment: 0: PRID; 1: PRID + 500 IU or 750 IU eCG

CI:Confidence Interval

The third binary logistic regression indicated significant effects of bull and herd on the PRATE28-34. Based on the odds ratio, the likelihood of being pregnant following treatment was lower by a factor of 0.37 in the herd 2 cows compared to the cows in herd 1 (Table 6).

Table 6. Odds ratios of the variables included in the final logistic regression model for factors affecting the conception rate following treatment in cows showing a long anoestrous period.

Factor	Class	N	% Conception rate	Odds Ratio	95% CI	P
AI Bull	12 classes					0.02
	Bull 1	9/18	50.0	9	1.5-50	
	Bull 2	13/25	52.0	9.75	1.8-51	
Herd	1	68/211	32.2	Reference		
	2	26/52	50.0	0.37	0.2-0.7	0.02

P=0.0001 Hosmer and Lemeshow Goodness-of-fit test = 21.6; 3 d

CI:Confidence Interval

Finally, no significant effects of the variables examined were observed on PLOSS in pregnant cows.

4. DISCUSSION

A dose of eCG added to a progesterone-releasing device fitted from 51 to 57 days postpartum did not improve the reproductive performance of our high-producing dairy cows compared to the use of a PRID alone, with the exception of cows showing a lack of luteal tissue at the start of treatment. In contrast, the addition of eCG to the oestrus synchronizing protocol was found to be beneficial in cows showing a long anoestrus period, greatly increasing the rates of both oestrus and ovulation. No differences were found between 500 UI and 750 UI eCG groups, according Kenyon et al. (2012).

In a previous study, we observed that progesterone treatment during the postpartum period improved the oestrus rate (Garcia-Ispuerto et al. 2010). The postpartum cow suffers a negative energy balance during early lactation reflected by a lowered body condition score that leads to diminished plasma progesterone levels (Santos et al. 2001). It has been hypothesized that changes observed in the reproductive physiology of cows under highly intensive management could be the outcome of a high rate of steroid metabolism (Wiltbank et al. 2006). Although in beef cattle eCG treatment improves pregnancy rate in cows with low body condition score (Souza et al, 2009; Sales et al, 2011), in the high producing dairy cow maybe has it is possible that progesterone supplementation in the absence of eCG at this time will enhance oestrus behaviour *per se* (Roelofs et al. 2010) due to the high steroid clearance rate. In contrast, eCG is needed in cows suffering a long period of anoestrus. In effect, all the present cows experimenting a long anoestrus period treated with eCG showed similar oestrus and ovulation rates even during the warm period. Probably, when the energy balance is restored, eCG supplementation improves oestrus and ovulation rates due both to its FSH- and LH-like activity (Bever et al. 1989) and treatment is capable of overcoming heat stress.

Cows 51-57 days into the postpartum period treated with the PRID during the warm period returned the highest oestrus rates in this study. In our geographical zone, heat stress is one of the most important factors affecting reproductive performance (López-Gatiús 2003; Garcia-Ispuerto et al. 2007). The oestrus rate in the warm period is normally impaired due to an increase in silent ovulations (Gwazdauskas et al. 1981; De

Rensis and Scaramuzzi 2003; López-Gatius et al. 2008). Thus, the question that arises is how did progesterone supplementation serve to increase the expression of oestrus during the warm period in cows treated with a progesterone-releasing intravaginal device alone compared to all the other groups? Plasma oestradiol concentrations are reduced by heat stress in dairy cows (Wolfenson et al. 1995, 1997). However, more controversy exists as to the effect of heat stress on plasma progesterone concentrations (Trout et al. 1998; Ronchi et al. 2001; De Rensis and Scaramuzzi 2003). If the concentration of plasma progesterone is reduced by heat stress this will have negative consequences on the expression of oestrus and fertility rates, and this easily explains the positive effects of progesterone treatment during periods of heat stress. Thus, it seems that the beneficial effects of progesterone on the expression of oestrus (Roelofs et al. 2010) are potentiated in conditions of heat stress.

In our study, cows expressing oestrus before 51–57 days postpartum showed a 1.45 and 1.6 times greater likelihood of oestrus and early conception in response to treatment in agreement with previous findings (Garcia-Ispuerto et al. 2010). In contrast, cows that do not return to oestrus before 56 days postpartum, have a lower conception rate (Yamada et al. 2003; Santos et al. 2004; Stevenson et al. 2006; Yáñez et al. 2006). Thus, cows that quickly resume oestrus following parturition, i.e., cyclic cows, are more sensitive to treatment, probably owing to the presence of a corpus luteum in the ovary (Sá Filho et al. 2010). Given that in high-producing dairy cows first postpartum ovulation is often delayed, methods designed to induce cyclicity in anoestrous cows should improve their reproductive performance (Yáñez et al. 2004; Rostami et al. 2011). In this study, cows with no luteal tissue at the beginning of treatment on Days 51–57 postpartum not receiving eCG showed a 2.5 (1/0.4) times lower conception rate on Day 120 postpartum compared to the other groups. This reinforces the idea that eCG is a useful tool in anoestrous cows. Thus, gynaecological exams including rectal palpation or ultrasonography are needed to determine the usefulness or not of adding eCG to a progesterone-releasing device protocol.

Cows with 2 or more corpora lutea 12 days after PRID removal (approximately 10 days post-AI) were 2.4 times more likely to conceive than cows with a single corpus luteum. Although fertilization rates in cattle are reported to be greater than 90% (Diskin and Sreenan 1980), most embryonic mortality (70–80% of total losses) occurs 8–16 d

after AI (Dunne et al. 2000; Sreenan et al. 2000). Thus, increasing the ovulation rate, as naturally occurs in older cows (López-Gatius et al. 2004), might be a strategy for cows and probably other mammals to increase the chances of pregnancy. In high-producing dairy cows in which reproductive performance is impaired yet management practices and nutrition are normally optimal, it is possibly that natural selection forces will drive double ovulation as a mechanism to improve fertility.

In conclusion, the administration of eCG in addition to an intravaginal progesterone-releasing device was beneficial in anoestrous cows even during the postpartum period (only in cows with no luteal tissue on days 51-57 postpartum). No differences were detected between the eCG doses 750 IU and 500 IU in both experiments such that we recommend 500 IU of eCG given on the day of PRID removal. Irrespective of the treatment, cows undergoing double ovulation were more fertile than the remaining cows.

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GENERAL DISCUSSION

GENERAL DISCUSSION

The main scope of this thesis was to improve reproductive performance of high producing dairy cows by establishing an ultrasonography based monitoring program on Days 15-21 postpartum and by implementing a progesterone-based synchronization protocol plus eCG in anestrous cows.

1. Peripartum influence on reproductive performance.

Parturition is an intricate and traumatic procedure that may condition subsequent lactation. After calving, cow has to return to its initial non-pregnant status. Postpartum recovery is a complex duel where immune system faces bacterial contamination (Hussain 1989; Singh *et al.* 2008). Whether the cow cannot overcome the infection, the process will be delayed and reproductive performance would be impaired (LeBlanc 2008, LeBlanc *et al.* 2012). In our studies, a negative effect of calving complications, such as placenta retention, dystotic parturition, stillbirth or previous twin pregnancy on subsequent conception rate was observed (Chapter 2). In agreement with previous reports (Grön *et al.* 1990; Fourichon *et al.* 2000; Mee 2008; López-Gatius *et al.* 2006; Bell and Roberts 2007), these peripartum events were associated to an impaired fertility, probably due to an amplified inflammation response that may delay uterine involution. Moreover, cows with antibiotic requirement on first week postpartum and those that had abnormal uterine involution on Days 29-35 postpartum had worse reproductive performance than the remaining animals (Chapter 2 and 3). Because of ineffectiveness of proposed treatments, it seems that peripartum events are determining not only the reproduction of the cow, but also its productivity. For that reason, preventing parturition complications should be a main goal of dairy herds.

1.1 Ovary-uterus crosstalk

Postpartum involution is a dynamic and multifactorial process where uterus and ovary are intimately connected to each other. Uterine infection can disturb ovarian folliculogenesis (Sheldon *et al.* 2002; Sheldon and Dobson 2004) whereas reproductive

endocrine environment created by the ovary can modulate the local immune response (Frank *et al.* 1984; Lewis 2004), predisposing the animal to disease or recovery. A complex debate has been performed along the years regarding the possible benefits of an early resumption of ovarian activity (Stevenson and Call 1983; Olson *et al.* 1984). In this thesis, a positive effect of return to ovarian cyclicity has been revealed (Chapter 2 and 4). Although a negative effect of progesterone on uterine defenses is expected (Lewis 2004; Singh *et al.* 2008), cyclic animals during the early postpartum period had a better subsequent reproductive performance. Perhaps, the positive effect of estrogens associated to estrus and ovulation (Hussain 1989) is enough to enhance immune response and facilitate postpartum involution. In fact, prior estruses to the moment of synchronization have been demonstrated to improve subsequent fertility (Garcia-Ispierto *et al.* 2010). Thus, it is logical to think that estradiol administration would help postpartum recovery. However, Sheldon *et al.* (2003) showed no effect of the addition of exogenous intrauterine estradiol on its involution. It is therefore suggested that postpartum uterus immunomodulation is not completely determined by endocrine regulation, and that unknown internal mechanisms should be involved.

1.2 Defining endometritis.

A basic principle in veterinary medicine is that “the earlier one can diagnose a sick animal and provide care, the faster that animal will return to a normal state of health” (Smith and Risco 2005). However, sometimes clinicians just see the tip of the iceberg. Endometritis, has a clear impact on reproduction (LeBlanc *et al.* 2002, Gilbert *et al.* 2005) but its diagnosis is still under discussion (Lewis 1997; Barlund *et al.* 2008; Senosy *et al.* 2009). Since 2006 (Sheldon, 2006), the definition of endometritis involved animals with presence of purulent uterine discharge detectable in the vagina 21 days or more postpartum. The same definition but before Day 21 may include a proportion of animals that will spontaneously resolve bacterial contamination (Sheldon *et al.* 2006). However, although this statement is possibly close to the reality, would it not be possible that this early inflammation or abnormal involution could impair subsequent reproductive performance even in spontaneous resumption? Our results showed a clear yes. A significant effect of clinical findings on Days 15- 21 postpartum on subsequent reproductive performance was registered (Chapter 1). Thus, the early identification of abnormal uterine involution will allow an appropriate treatment of cows

Finding an economical-effective technique to detect cows suffering from endometritis is another challenge for dairy herds. Although uterine biopsy is the gold standard method (Bonnett *et al.* 1991; Chapwanya *et al.* 2009), it is generally quite far to be implemented at clinical level. Traditional vaginal and transrectal examination are economic techniques, but overlook important information about uterine health status (LeBlanc 2008) (Chapter 1). Therefore, the implementation of other techniques in postpartum examination is necessary for a proper diagnosis. Endometrial cytology has been used for diagnosing endometritis after Day 21 postpartum (Kasimanickam *et al.* 2004; Dubuc *et al.* 2010; Dourey *et al.* 2011). However, its usefulness on the previous weeks has not been proved yet. In fact, our results showed no conclusive association between cytological findings and subsequent reproductive performance (Chapter 1). Furthermore, transrectal ultrasonography is an economical and fast method already implemented on the reproductive control of dairy herds. Measuring endometrial thickness, cervical diameter and determining the presence of intrauterine fluid (IUF) were good markers for predicting subsequent reproductive performance in postpartum cows (Chapter 1). Thus, postpartum ultrasound examination of the genital tract may emerge as a routine monitoring technique for diagnosing endometritis.

1.3 Efficiency of current postpartum treatments

The use of prostaglandin $F_{2\alpha}$ in postpartum treatments is widely used in veterinary medicine. However, even with its theoretical advantages on postpartum involution (Hussain and Daniel 1991; Lewis 2004), in practice its effectiveness is still under discussion (López-Gatius and Camón-Urgel 1989; Bonnett *et al.* 1990; Etherington *et al.* 1994; Hendricks *et al.* 2006; Galvão *et al.* 2009; Kaufmann *et al.* 2010; Dubuc *et al.* 2011; Salasel and Mokhtari 2011). Our results showed no effect of prostaglandin on the third week postpartum, even in cyclic cows (Chapter 2). The uterus produces endogenous prostaglandin after parturition and is increased in cows with endometritis (Del Vecchio *et al.* 1994; Mateus *et al.*, 2003). Thus, the effect of providing more prostaglandin to these animals seems not to be advisable.

Another commonly used therapeutic protocol is systemic or local antibiotic treatment. Bacterial etiology of postpartum uterine disease is dynamic and multifactorial

(Bicalho *et al.* 2012). Thus, knowing the etiological origin of the infection-whenver possible- would help to the election of an appropriate antibiotic. A co-infection of both, *Arcanobacterium pyogenes* and *Fusobacterium necrophorum* is possibly the responsible of most postpartum endometritis (Ruder *et al.* 1981; Dohmen *et al.* 2000). Therefore, a broad-spectrum antibiotic should be used during the third week postpartum (Azawi 2008). For veterinarians, it is sometimes difficult to lead to an already established protocol in dairy herds. This was the reason why penicillin, a Gram negative effective antibiotic, was used in chapter 3. Our results indicated no effect of intrauterine penicillin on Days 15-21 postpartum on uterine involution or conception rate. Thus a modification of the treatment protocol, including broad-spectrum antibiotic lavage, was suggested.

Antibiotic resistance in humans and animals is an increasing concern worldwide (Witte 1998; Okeke *et al.* 2005). Therefore, as alternative treatment, uterine lavages with physiologic saline serum were proposed for evacuating uterine fluids without use of any type of drug. This technique is frequently used in mares (Pycock 2007; Brinsko *et al.* 2011), providing successful results in reducing intrauterine bacterial density and endometrial inflammation (Troedsson *et al.* 1995). However, no significant effect was observed in cows on subsequent reproductive performance (Chapter 3). Probably, more lavage volume is needed to achieve a correct evacuation of IUF. Further research is required to assess the utility of uterine lavage with physiologic saline serum on postpartum dairy cows.

2. Pathway to pregnancy. Progesterone-based estrus synchronization protocol.

Once a cow has successfully undergone postpartum involution, it should be enrolled in a management program to conceive again. The use of progesterone intravaginal devices to synchronize the estrus has been demonstrated to be effective (Garcia-Ispuerto *et al.*, 2010) after uterine involution. The protocol performed in Chapter 4, was a modified progesterone-based estrus synchronization protocol. An equine chorionic gonadotrophin (eCG) was added at device removal.

The hormone eCG is frequent used in sheep and beef cows for inducing superovulation (Bo *et al.* 2003; Amiris and Cseh *et al.* 2012; Kenyon *et al.* 2012) and normally it is combined with estradiol in synchronization protocols (Sá Filho *et al.* 2010), not allowed in EU. Its effect on reproductive performance in a synchronization protocol in dairy cows had not been tested yet. This new protocol not only counteracts the detrimental effect of the warm period on estrous behavior, but also induced ovulation in long anoestrus cows (Chapter 4). Although no effect was seen in fertility, total number of animals inseminated increased in eCG treated animals compared to the remaining cows (Chapter 4). Implement this protocol on fixed time AI routine, should be then, the focus of further studies.

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CONCLUSIONS

CONCLUSIONS

The main conclusions of this thesis are:

- Measuring cervical diameter, endometrial thickness and detecting the echogenicity of IUF by ultrasonography from Days 15 to 21 postpartum is a good tool to predict subsequent reproductive performance in clinically normal dairy cows.
- Vaginal examination and transrectal palpation alone on Days 15-21 postpartum are not valuable predictors of subsequent reproductive performance.
- No effect of treatment with PG on Days 15-21 postpartum was observed on subsequent reproductive performance of high producing dairy cows, even in cyclic animals.
- Early resumption of ovarian cyclicity enhances postpartum recovery and improves subsequent conception rate.
- No effect was observed of a single intrauterine infusion of penicillin or normal saline serum on Days 15-21 postpartum of cows suffering endometritis on the subsequent conception rate or return to cyclicity.
- Calving season was intimately related to reproductive performance.
- Administration of eCG in addition to an intravaginal progesterone-releasing device was beneficial in anoestrous cows even during the postpartum period (only in cows with no luteal tissue on days 51-57 postpartum).
- No differences were detected between the eCG doses 750 IU and 500 IU in both experiments
- Cows undergoing double ovulation after eCG-progesterone-based synchronization protocol were more fertile than the remaining cows.

EPILOGUE

FURTHER ASPECTS ON CLINICAL PERSPECTIVES

Understanding of postpartum physiology is quite rudimentary, despite extensive research efforts made along years. Unfortunately, our knowledge about pathophysiology of the diseased postpartum uterus is even more deficient. That leads to unreliable information about postpartum status of the farm that can impair subsequent fertility. With a better understanding of postpartum uterus and immune response, researchers will be able to suggest better diagnostic tools and, eventually, a more efficient therapeutic approach to deal with uterine pathology.

PREVENTING UTERINE DISEASE

Cows suffer dramatic changes in energy balance during the peripartum period. In the last month of gestation, the energetic requirements rise while dry matter intake decreases. Thus, animal has to deal with a negative energy balance (NEB) (Goff and Horst 1997). Therefore, prepartum diets are recalculated to guarantee energy and nutrients demands (McNamara *et al.* 2003). After parturition, energetic requirements are still elevated due to milk production and the NEB can be extended along months particularly in high producing dairy cows (Dobson *et al.* 2007). This energy and nutritional deficit has been related to immune depression, evident in postpartum cows (Hammon *et al.* 2006; Goff 2008). Whether NEB is reduced, metabolic (milk fever, ketosis, retained placenta, displaced abomasum) and infectious (mastitis, metritis, endometritis) postpartum diseases will be diminished (Mulligan *et al.* 2006; LeBlanc 2008), reducing its impact on subsequent reproductive performance (Roche 2006).

A valuable predictor of NEB is the loss of body condition score (BCS), an internationally accepted subjective visual and tactile measure of body condition (López-Gatius *et al.* 2003). Monitoring pre- and postpartum BCS is an aid to take nutritional and management decisions in order to ensure a mild, but not severe NEB in early postpartum and to minimize its carry-over effects into the remain lactation (Markusfeld *et al.* 1997; Walsh *et al.* 2011). Moreover, measuring metabolic markers such as nonesterified fatty acids or β -hydroxybutyrate produced by an overload hepatic function in a NEB (Goff and Horst 1997), could also be an appropriate future prevent and

diagnostic tool to detect unhealthy postpartum cows (Ospina *et al.* 2009; Bjerre-Harpøth *et al.* 2012).

Parturition is a critical moment that will determinate the postpartum evolution and the subsequent reproductive performance of the cow. Veterinarian interventions at dystotic calvings can be sometimes solved by hand manipulation (Norman and Youngquist 2007). However, this human participation frequently ends in aggressive traction and commonly leads to surgical procedures that can comprise uterine health (Mee 2008). At the same time, hygienic conditions of the farm seem to be important for postpartum uterine infection. However, some studies demonstrated that hygienic conditions could not be as important as it was thought (Noakes *et al.* 1991). Probably, despite the severity of uterine bacterial contamination, individual immune response is the key point to maintain uterine health.

Future research should be focus on enhancing immune system specially depressed during postpartum period (Singh *et al.* 2008; LeBlanc *et al.* 2011). This medical area is particularly being developed in human cancer medicine and emerges as a present and future feasible therapy (Talmadge 2002). Improving cow immune response, not only innate but also humoral and cell-mediated immunity during peripartum period, would mitigate uterine diseases (Dhaliwal *et al.* 2001; Nolte *et al.* 2001).

DIAGNOSING ENDOMETRITIS

Different diagnosis strategies have been developed to detect postpartum animals with a subsequent impaired fertility. Endometrial biopsy is now the most accurate method to identify endometrial inflammation (Messier *et al.* 1984; Bonnett *et al.* 1991). However, other strategies such as uterine prostaglandin $F_{2\alpha}$ -release (as inferred from plasma 13,14-dihydro-ketoprostaglandin $F_{2\alpha}$ concentrations; PGFM) may also provide information about uterine and cervix involution (Lindell *et al.* 1982). This method simplifies cow management at farm level compared to biopsy. PGFM is generally high during and immediately after parturition and it declines to basal concentration by Day 20 postpartum (Kindahl *et al.* 1992). Recent studies relate increased levels of PGFM on Days 21-28 postpartum to uterine infection (Del Vecchio *et al.* 1994; Seals *et al.* 2002),

detecting unhealthy animals with reproductive disorders. Although these laboratory determinations are potentially a new practical diagnostic tool, to the best of our knowledge, nowadays there is no quick test to determine PGFM concentrations in herd. Therefore, after this thesis research, as far as we concern, ultrasonography appears as a routine and accurate technique to detect cows with endometritis in dairy herds.

TREATING ENDOMETRITIS

Conventional protocols established in the herds probably are not the most effective option. Thus, validate treatment protocols with internal test experiments can provide information about its utility. Prostaglandins at Days 15-21 postpartum have shown no effect on subsequent fertility on cows suffering endometritis (Chapter 2). Therefore, what should a clinician do when an endometritic cow is detected? Is there any proficient therapeutical option? Nowadays, the only commercially approved product for intrauterine administration in cows with endometritis contains benzathine chepapirin (NRA 2001). Although positive effects of cephapirin have been described (LeBlanc *et al.* 2002; Kasimanickam *et al.* 2005), number of studies supporting its efficiency is quite poor (Léfebvre and Stock 2012). Several combinations have been tested to treat endometritis: antibiotics (LeBlanc 2008), estradiol (Sheldon *et al.* 2003), intrauterine enzymes (Drillich *et al.* 2005), and even homeopathic remedies (Arlt *et al.* 2009). However, no positive and convincing results have been shown. Further work should be done in searching an effective endometritis treatment for diminishing its effect on reproductive performance.

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APPENDIX

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CURRICULUM VITAE

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Investigador en formación de Proyecto en la Universidad de Lleida (UdL)
Departamento de Producción Animal. Centro de Investigación en Producción Animal (CIPA). Escuela Técnica Superior de Ingeniería Agraria. Fecha de inicio: 01/10/2009

Estudios de doctorado:

- Línea de investigación “Factores que afectan a la eficiencia reproductiva del ganado vacuno lechero”.
- Título de tesis: “**From parturition to pregnancy. A clinical perspective in the dairy cow.**”
- Directores: Dr. F.López-Gatius y Dra. I.García-Ispuerto.

- **Formación académica universitaria**

-Máster Universitario en Biotecnología en Ciencias de la Salud
Otorgado por la Universidad de Lleida. Septiembre 2010.

-Licenciatura en Veterinaria

Otorgado por la Universidad Complutense de Madrid. Octubre 2008.

- **Experiencia científica y tecnológica**

Participación en proyectos de I+D+i

-Interacciones inmunoendocrinas materno-fetal y con Coxiella burnetii en vacas infectadas con Neospora caninum. Efecto del tratamiento con melatonina en ambas enfermedades. Investigador responsable: Fernando Román López Gatius. Entidad financiadora: Ministerio de Ciencia e Innovación.AGL2010-21273-C03/GAN. Fecha de inicio: 2010, 2 años

-Grupo consolidado sobre factores que afectan a la fertilidad y al mantenimiento de la gestación en vacuno lechero. Investigador responsable: Fernando Román López Gatius. Entidad/es financiadora/s: Departamento de Innovación, Universidades y Empresas. 2009 SGR 816. Fecha de inicio: 2009, 4 años

Participación en contratos

-Eficiencia reproductiva en el vacuno lechero de alta producción tratado con PRID durante el periodo postparto. Investigador responsable: Fernando Román López Gatius. Entidad/es financiadora/s: CEVA Santé Animale. C06073. Fecha inicio: 2006, 6 años

- **Producción científica**

Artículos científicos.

-Reproductive performance of anoestrous high-producing dairy cows improved by adding equine chorionic gonadotrophin to a progesterone-based oestrus synchronizing

protocol. Garcia-Ispierto I, López-Helguera I, Martino A, López-Gatius F. *Reproduction in Domestic Animals*. Doi: 10.1111/j.1439-0531.2011.01954.

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-Single administration of PGF2 alpha on days 15-21 postpartum failed to improve subsequent fertility of dairy cows. Lopez-Helguera I.; Lopez-Gatius F.; Andreu-Vazquez C.; Garcia-Ispierto I. *Reproduction in Domestic Animals*, vol. 46, (Suppl. S3), p. 124 (2011) Online ISSN: 0936-6768.

-Prepartum Lymphocytic counts are positively correlated to Somatic Cell count in the early lactation. I, López-Helguera; I. Garcia-Ispierto; A. Abdelfatah-Hassan; F. López-Gatius. *Reproduction in Domestic Animals*, vol. 45, (Suppl. s2), p. 84 (2010) Online ISSN: 1439-0531.

-The effect of addition of equine chorionic gonadotropin to a progesterone-based estrous synchronization protocol in postpartum anestrous dairy cows. I.Garcia-Ispierto; I. López-Helguera; S. Floch; A.Martino; F. López-Gatius. *Reproduction in Domestic Animals*, vol. 45, (Suppl. s2), p. 84 (2010) Online ISSN: 1439-0531.

Trabajos presentados en congresos nacionales o internacionales

-Effects of addition of eCG to a progesterone-based estrous synchronization protocol on subsequent ovulation and conception rate in postpartum dairy cows. I. López-Helguera; F. López-Gatius; C. Andreu-Vázquez; I. Garcia-Ispierto. Póster. XXVII World Buiatrics Congress 2012.

-Effects of addition of eCG to a progesterone-based estrous synchronization protocol on subsequent estrous and conception rate in long anestrous dairy cows. I. López-Helguera; F. López-Gatius; C. Andreu-Vázquez; A.Martino; I. Garcia-Ispierto. Póster. XXVII World Buiatrics Congress 2012

-Control ecográfico de la gestación. El problema de las gestaciones gemelares. F. López-Gatius; C. Andreu-Vázquez; I. López-Helguera; I. Garcia-Ispierto. Ponencia. XVII congreso Internacional ANEMBE de Medicina Bovina 2012.

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-Evaluación clínica de la involución uterina: aspectos hematológicos y ecográficos. I. López-Helguera; I. Garcia-Ispierto; F. López-Gatius. Comunicación oral. XV congreso Internacional ANEMBE de Medicina Bovina 2010.

-Importancia de la necropsia como método diagnóstico y herramienta para la prevención de enfermedades en cebaderos. A propósito de un caso clínico. I. López-Helguera; M. Rodríguez Martín. Póster.VII Congreso de Ciencias Veterinarias y Biomédicas 2008.

-Tratamiento farmacológico de la queratitis ulcerativa en la clínica equina. I. López-Helguera; M. López Montero. Comunicación oral.V Congreso de Ciencias Veterinarias y Biomédicas 2006.

-Staphylococcus aureus resistente a meticilina en perros y gatos, un problema emergente. I. López-Helguera; L. Hidalgo del Rio; B. Gutiérrez Soriano. Comunicación oral 2006.

-El enemigo en casa: los microorganismos como transmisores de resistencia a antibióticos. P. Limón Sánchez; I. López-Helguera; M. Pereira Martín. Comunicación oral. IV Congreso de Ciencias Veterinarias y Biomédicas 2005.

- **Docencia impartida**

Curso académico 2010-2011: Impartición de un total de **32 horas** de docencia práctica en Fisiología Animal I, Anatomía Animal I y Reproducción Animal en el grado de Ciencia y Salud Animal (Udl).

Curso académico 2011-2012: Impartición de un total de **20 horas** de docencia práctica en Anatomía Animal I y Reproducción Animal en el grado de Ciencia y Salud Animal (Udl).

Cursos de Postgrado Recibidos:

-Curso de formación para el personal investigador con animales de experimentación Otorgado por la Unidad de Formación del Profesorado Universitario-Universidad de Lleida. Octubre 2010.

-Curso de Adaptación Pedagógica Otorgado por la Universidad Complutense de Madrid. Enero 2009

- **Experiencia laboral**

-Desde Octubre 2009: Control y asesoramiento reproductivo en granjas lecheras de alta producción. Lleida. Manejo de técnicas en campo: citología y biopsia uterina, ecografía modo B y modo Doppler. Colaboración en transferencias de embriones.

-Diciembre 2008-Septiembre 2009: Contratada en clínica veterinaria de pequeños animales “clínica veterinaria parque de la ventilla” Madrid. Área de trabajo: medicina y cirugía general.

-Septiembre 2007-Agosto 2008: Estancias de prácticas en “Trialvet Asesoría e Investigación Veterinaria” Madrid. Área de trabajo, vacuno: medicina general, control reproductivo, control lechero y colaboración en mataderos.

-Julio 2007-Agosto 2007: Estancias de prácticas con un veterinario de medicina ovina, Albacete. Área de trabajo: medicina general, trabajo de ADS.

-Septiembre 2005-Junio 2007: Estancias de prácticas en “Fundación ONCE del perro guía” Madrid. Área de trabajo: reproducción canina, medicina y cirugía general, radiología y control epidemiológico del centro.

Live together, die alone.

Jack Shephard