Universitat de Lleida

Factors of Non-Infectius Nature Affecting Late Embryonic/Early Foetal Loss in High Producing Dairy Cows: A Therapeutic Approach

Gregori Bech Sàbat

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Cover picture: Doppler-7,5 MHz-Ultrasonograph of a 35-day bovine embryo.
Back cover picture: Pink Floyd's "Atom Heart Mother" Album Cover.

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Pel meu avi.

Encara que no acabés d'entendre que als 28 anys seguís "estudiant", segur que li hagués agradat poder estar a la defensa. Per poc temps no ha pogut ser.

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1 GENERAL INTRODUCTION

1.1 Introduction

Nature provided to human ancestors food to survive. With time, they became agriculturalists and farmers. In fact, regulating nature cannot be overvalued within the human culture. Nowadays, although economically not a major player, the livestock sector is socially and politically very significant. Livestock production accounts for 70% of all agricultural land, which means that it takes up 30% of the ice-free land on the planet (Steinfeld *et al*., 2006). It employs 1300 million people and creates livelihoods for another 1000 million (Steinfeld *et al*., 2006). And that is just the current situation. Growing populations and incomes, along with changing food preferences, are rapidly increasing demand for livestock products. Thus, although in "developed" countries consumption of livestock products is stagnating and markets and people are saturated, dramatic developments in rapidly growing developing countries are occurring (FAO, 2006).

Within animal productions systems, ruminants have a main role (Church, 1993), and cattle is one of the ruminant species more used by humans. Cattle were domesticated probably 8500 years ago in the Middle-East (McGee, 2007). Rock drawings from the Sahara show that dairying was known by 4000 BC, and what appear to be the remains of cheese have been found in Egyptian tombs dating back to 2300 BC (McGee, 2007). References on milk can be found in many historical texts, for example on Aristotle' *On the generation of animals*, or the *Bible*. Even the galaxy where we belong has been named the Milky Way. However, the general use of milk and milk products as food has come about only with the development of modern societies (Eckles, 1911). Dairying is probably the most efficient form of animal production and has long been synonymous with wholesome, fundamental nutrition (Eckles, 1911; McGee, 2007). Nevertheless, over the last few decades, the idealized portrait of milk has become more shaded, because of lactose-intolerance and fat contents. But it is, and probably will be for a long time, a basic product in human diet. Moreover, healthy issues can also be linked to milk (Calsamiglia, 2008) and dairy foods have been identified as an important part of a potential strategy to alleviate food insecurity in regions like USA (Palacios *et al*., 2007).

The Food and Agriculture Organization estimated a population of 1347 millions of heads of cattle in the world in 2008, including 247 millions of heads of dairy cattle producing almost 600 million tonnes of milk (FAOSTAT, 2010). Furthermore, milk production is projected to be around 1000 million tonnes on 2050 (Steinfeld *et al*., 2006). The European Union (EU-27) production represents 22% of the world production (133 million tonnes milk), with a downward trend in dairy cow numbers (but still accounting almost 24 million heads in 2007) coupled with an increased milk yield per cow (EUROSTAT, 2009). In Spain the picture is similar. In the early nineties, almost 140000 herds with 1.5 million milking cows were producing around 6 million tonnes of milk. In 2008, Spain was still producing the same amount of milk, but number of herds descended to 24000 with 900000 milking cows (MARM, 2010). Moreover, the situation is highly polarized, with two models of production, a semiextensive one and another highly intensified.

Since the Industrial Revolution in the XVIIIth century, many countries of the world have suffered a high industrialization of the processes of production. During the last century it has been also the turn for some animal production systems, emerging a new type of ecosystems, which have been defined as "bioindustrial ecosystems" (Brander and Cole, 1986). It has been the case of dairying in many regions (Line, 1986). Rapid progresses in management and genetics have increased significantly milk production and number of animals per herd, transforming completely the manners of production.

In dairy cattle herds, and especially in high production systems, reproduction is a cornerstone for economic success. Reproduction is timely organizing the herd: milk production of individual cows depends on their ability to become pregnant and maintain gestation, because lactation is initiated only after parturition. In recent decades, along with increasing milk production, it has been widely reported a dramatic decrease in reproductive performance, accurately described (Royal *et al.*, 2000b; López-Gatius, 2003) and reviewed (Lucy, 2001; Dobson *et al.*, 2007; Lucy, 2007). The reason is not only selection for milk production. A combination of a variety of physiological, environmental and management factors have an additive effect on reproductive efficiency. One challenge that is undoubtedly affecting efficiency of the dairy industry is the decline in fertility and reproductive efficiency in modern dairy herds.

As a broad concept, fertility in a female mammal is defined as the ability to conceive and maintain pregnancy producing viable offspring (Yániz *et al.*, 2008). Getting the cow pregnant in a reasonable time is an important issue and primary attention is usually directed towards a failure in correctly detecting oestrus, the inappropriate timing of pregnancy and postpartum reproductive health. Despite progresses in reproductive and management procedures, fertility is decreasing year after year. The annual rate of decline in fertility was 0.45% in the USA expressed in terms of pregnancy rate (Butler and Smith, 1989; Beam and Butler, 1999), 0.75% in Ireland (Mee, 2004) and 1% in the UK (Royal *et al.*, 2000a,b) expressed in terms of conception rate to first service. In Spain, the annual decline in pregnancy rates was 0.5% per annum between 1991 and 2000 (López-Gatius, 2003).

However, once the cow is pregnant, it is also essential that pregnancy progresses safely to term. Pregnancy failure has been described as the major single source of economic losses sustained by dairy producers (Ball, 1997). The majority of the research on pregnancy loss has focused on causes of embryonic deaths that occur before maternal recognition of pregnancy. In comparison to conception rate and early embryonic mortality, less research has been devoted to the problem of late embryonic and foetal mortality and less characterized are the factors that affect this reproductive parameter. With the advent of ultrasonography and pregnancy-associated glycoprotein determination, accurate pregnancy diagnosis is possible as early as 26-28 days after AI in cattle (Pieterse *et al*., 1990; Zoli *et al*., 1992; Szenci *et al*., 1998a; Fricke, 2002; Sousa *et al*., 2008), thereby facilitation the study of embryonic mortality after the period of maternal recognition of pregnancy.

1.2 Early Foetal Loss

1.2.1 The embryonic and early foetal period[1]

In mammals, pregnancy is the period during which a female carries the offspring inside the genital tract, starting at conception and finishing with calving. The gestation period in the cow averages 282 days. It has been divided in an embryonic period that extends from conception to the end of the differentiation stage (about 42 days), and a foetal period that runs from day 42 to parturition (Committee on Bovine Reproductive Nomenclature, 1972).

During the first week of development the embryo starts a series of mitotic division together with the correct genome activation and establishment of the first lineage decisions leading to trophectoderm and inner cell mass. By day 4-5 it has already reached the uterus. During the second week of bovine development gastrulation is orchestrated and the free-floating embryo within the lumen of the uterus starts a massive growth, developing the extraembryonic membranes, mainly from the trophectoderm (Vejlsted *et al*., 2006). Conceptus (the embryo and the extraembryonic membranes) must provide a timely biochemical signal to the mother, or pregnancy will terminate because a new oestrous cycle will begin. This process is known as maternal recognition of pregnancy, involving embryonic-maternal signalling, with a main role of IFN-τ produced by the trophoblastic cells (Bazer *et al*., 1986; Thatcher *et al*., 1997; Robinson *et al*., 2008). The critical period is the 15^{th}-16^{th} days after ovulation (Inskeep and Dailey, 2005), continuing towards the beginning of the process of attachment of the extraembryonic membranes to the endometrium by the end of the third week (Guillomot, 1995; Imakawa *et al*., 2004). At this stage, the embryo has three body layers and rudimentary organs, as well as extraembryonic membranes. The process of attachment is not completely finished till, at least, day 47 (Riding *et al*., 2008).

Attachment of the conceptus to form the placenta, an intimate, but temporary, relationship with the uterus, is the result of a long evolutionary way that provides significant advantage to the conceptus, ensuring nutrition and metabolic exchange as well as protection. The term "implantation" is often used to mean attachment of the placental membranes to the endometrium. Actually, true implantation only occurs in rodents and humans (Cross *et al*., 1994; Wang and Dey,

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[1] For further information in this section, please see (Hafez and Hafez, 1980; Hunter, 1982; Santolaria Blasco and Yániz Pérez de Albéniz, 2001; Senger, 2003; Ball and Peters, 2004)

2006), which conceptus buries itself into uterine endometrium. In the cow (and most other domestic species) conceptus does not truly implant, but rather contacts intimately to the endometrium surface remaining in the luminal compartment.

In ruminants, the placenta is epithelio-chorial and cotiledonary (Wooding, 1992; Wooding *et al*., 1996). By "cotiledonary" it means that the chorionic villus (the functional unit of foetal placenta) form discrete button-like structures, called cotyledons, which are intimately joint with the maternal component derived from the uterine endometrium (the caruncular regions) forming units called placentomes (King *et al*., 1979; Atkinson *et al*., 1984; Laven and Peters, 2001). In cattle, approximately 100 to 150 functional placentomes constitute the fetoplacental unit. During gestation, cotyledons will increase many-fold in diameter providing an important area of contact (Schlafer *et al*., 2000). The term "epithelio-chorial" refers to the relationship between conceptus and maternal tissues: the endometrial epithelium (epithelio-) is in contact with the chorionic epithelium of the conceptus-side (-chorial). An important feature of the ruminant placenta is the formation of binucleate giant cells from the trophoblast cells, a unique cell type (Wooding and Wathes, 1980; Igwebuike, 2006). In the cow, these are formed from day 18-20 and are continuously formed throughout gestation. They migrate from the chorionic epithelium invading the endometrial epithelium transferring complex molecules from foetal to maternal placenta. Binucleate cells are important sites of steroidogenesis and endocrine sites, secreting the placental lactogens, which promotes foetal growth and stimulates the mammary gland of the dam, and the pregnancy-associated glycoproteins (PAG), which function remains still unknown, but they have been used for both pregnancy diagnosis and as a marker of foetal/placental well being (Skinner *et al*., 1996; Szenci *et al*., 1998a,b). Because the uterine epithelium is modified by invasion and fusion of binucleate cells, its structure is generally referred to as *synepitheliochorial.* Prior to detailed study of these structures, it was thought that the maternal epithelium was eroded away, leaving trophoblast in contact with maternal connective tissue. The term syndesmochorial was used to describe this apparent structure, and it is used in much of the older literature describing ruminant placentation.

Another important feature of the placenta is the development of the underlying vasculature. Adequate placental angiogenesis is critical for the establishment of the placental circulation and thus normal foetal growth and development (Reynolds and Redmer, 2001). The degree of placentomal formation increases until parturition, but in the first half of gestation there is already a highly elaborated fetomaternal villous-crypt exchange system (Pfarrer *et al*., 2001; Reynolds and Redmer, 2001).

1.2.2 Immunology of pregnancy

On 1953, Peter Medawar stated (Billington, 2003): *"The immunological problem of pregnancy may be formulated thus: how does the pregnant mother contrive to* *nourish within itself, for many weeks or months, a foetus that is an antigenically foreign body?".*

The statement points directly to the paradox of pregnancy: the conceptus, a semi-allograft, survives in the maternal organism. Moreover, during attachment and placentation, there is an increasing contact between the conceptus and maternal immune cells; however there is not (or should not be) rejection. Thus, successful pregnancy requires that the uterine mucosa tolerates the allogenic embryo without compromising its immune mechanisms against microbial agents (Fisher *et al*., 1985; Aagaard-Tillery *et al*., 2006; Davies, 2007).

It appears that different factors contribute to protection of the conceptus from immune-mediated rejection. First, the anatomical separation of the mother and foetus by the placenta isolates partly the two systems (Wooding, 1992; Schlafer *et al*., 2000). On the other hand, pregnancy is an immunosuppressive state permitting the foetal allograft to implant and grow (Fisher *et al*., 1985). Mechanisms leading to immunosupression include the downregulation of polymorphic major histocompatibility complex antigen expression on the trophoblast cells that form the external epithelial layer of the plancenta and a shift in the immunological ambient of the uterus (Bainbridge *et al*., 2001; Hill *et al*., 2002).

1.2.3 The biochemical basis and the role of progesterone

All these events for establishing and maintaining pregnancies in mammals are dependent upon a complex process of intimate conceptus-maternal crosstalk involving exquisite integration and coordination of several complex biological processes, including metabolic, endocrine, vascular, and immune functions (Cross *et al*., 1994; Vigano *et al*., 2003; Wolf *et al*., 2003; Spencer and Bazer, 2004; Spencer *et al*., 2007). A network of hormones, growth factors and cytokines govern pregnancy events. Mechanisms have evolved to ensure the continuity of species by redundant mechanisms and molecules involved being extremely pleiotropic (Vigano *et al*., 2003). Thus, the detailed picture of involved molecules and mechanisms is complex and still not completely elucidated. However, within all these molecules, progesterone has been for long identified unequivocally as a key character (Stormshak and Erb, 1961; Hansen, 1998; Spencer and Bazer, 2002; Spencer *et al*., 2004; Arck *et al*., 2007; Spencer *et al*., 2007).

Progesterone is crucial to establish and support a pregnancy. The biological activities of progesterone involve several modes of action (Mahesh *et al*., 1996; Falkenstein *et al*., 2000; Arck *et al*., 2007) which include genomic pathways, regulating gene expression mainly via nuclear receptors, and non-genomic pathways, affecting signal transduction cell responses mainly via plasma membrane receptors (Schmidt *et al*., 2000; Edwards, 2005; Bishop and Stormshak, 2008; Stormshak and Bishop, 2008; Gellersen *et al*., 2009). Although the cell and molecular mechanisms regulating early conceptus development and attachment are still not fully understood, it is known that progesterone and progesterone-dependent factors are essential for orchestrating the histotrophic environment necessary for conceptus growth and development (Bazer, 1975; Garrett *et al*., 1988; Geisert *et al*., 1988; Morris and Diskin, 2008). Implantation and placentation are also dependent on progesterone secretion (Spencer and Bazer, 2002; Spencer *et al*., 2004; Spencer *et al*., 2007).

Progesterone also plays a fundamental role in the immune-modulation of gestation, blocking T-cell lymphopoiesis during pregnancy and controlling the bias towards a pregnancy protective milieu (Hansen, 1998; Szekeres-Bartho *et al*., 2001; Druckmann and Druckmann, 2005; Arck *et al*., 2007).

1.2.4 Definition and rates of early foetal loss

The first trimester of bovine pregnancy is a critical period for the long-term development and viability of the foetus, highly sensitive to several forms of stress. A successful transition through day 90 constitutes an important milestone towards the birth of a healthy calf.

Most pregnancy losses in dairy cattle occur during the early embryonic period (Ball, 1997; Hanzen *et al*., 1999; Vanroose *et al*., 2000; Inskeep and Dailey, 2005) with a peak of losses on days 14-16 (Inskeep and Dailey, 2005). However, at practical level, these losses are included in indices as conception rate, because pregnancy diagnoses are only possible from day 20 onwards, and in most cases are not enough sensitive and specific till day 28, and usually are routinely performed on days 30-40 (Youngquist and Threlfall, 2006). Moreover very early losses cause minimal disruption to the breeding cycle and cows can return to oestrus, be inseminated and re-establish pregnancy with loss of time rarely exceeding the duration of two oestrous cycles. On the other hand, and as stated above, the development of the conceptus-maternal relationship is not established completely till approximately day 60, when gestation is considered firmly established, and chances of loss are reduced. In fact, in normal conditions without any infectious agent or exceptional situation, the risk of pregnancy loss after day 90 is minimal (López-Gatius *et al*. 2004).

From the two limits it can be established the type of losses studied in this thesis. There is a time frame between pregnancy diagnosis and the definitive establishment of gestation at day 60/90, where pregnancy losses can occur: the late embryonic and early foetal losses (or simply early foetal losses to simplify).

Earlier studies reported a basic level of loss during this period of approximately 5-8 % (Ball, 1997), perhaps representing inevitable genetic wastage as discussed by Bishop (1964). However more recent reviews show pregnancy losses after they have been diagnosed of 10-12 % (Santos *et al*., 2004; BonDurant, 2007). On the other hand, rates of losses are highly variable, and early foetal loss rates of more than 20 % have been reported (Cartmill *et al*., 2001a; Bartolome *et al*., 2005a-c; Grimard *et al*., 2006) or even 40% (Cartmill *et al*., 2001b), with increased risk under conditions of intensive management (Forar *et al*., 1995; Santos *et al*., 2004).

1.2.5 Economic importance of Early Foetal Loss

Compared with fertilisation failure and early embryo loss, more time is lost in the diagnosed pregnancy that is subsequently wasted, and in some cases there is a further loss of time after failure until the re-establishment of normal cycles and successful conception (Ball, 1997). It has been documented that additional days in which cows are not pregnant beyond the optimal time post-calving are costly (Olds *et al*., 1979; Groenendaal *et al*., 2004; Meadows *et al*., 2005). An often cited figure for the economic value of a pregnancy loss is the 640 $ (481 €) stated by Thurmond and Picanso (1990). This value result from a simple calculation, taking the approximately 4 $ for a day open (Olds *et al*., 1979) multiplied by 160 days lost (average 100 days of abortion + 60 days of rebreeding). Although a simple calculation, the latter is probably not far from the actual cost. In a recent study, De Vries (2006) focused on the economic value of pregnancy in dairy cattle. A mathematical model including many factors related with the cost of pregnancy loss, as persistency of lactation, probability of pregnancy, replacement heifer costs, milk prices, milk yield or culling decisions, resulted in an average cost of 555 $ (409 €) / pregnancy loss, reaching in some circumstances 1373 $ (1032 €) (De Vries, 2006). For example, a 4th-month pregnancy loss in a first-lactation cow producing around 12500 kg/year resulted in 993 $ (732 €) (De Vries, 2006). In another recent study, the gross economic loss for a pregnancy loss in dairy cows was up to 2333 $ (1721 €) including, basically, costs of days open and increased culling rates after pregnancy losses (Lee and Kim, 2007).

1.2.6 Causes of Early Foetal Loss

Prenatal losses can be caused by infections and by non-infectious factors. Primary attention has often been directed to infections but non-infectious causes probably account for the major part of the cases. Viral, bacterial, protozoal and mycoplasmal infections can result in embryonic and foetal mortality, indirectly by systemic effects on the dam, or directly by affecting the embryo or contaminating its environment. Depending on the agent they can prefer different stages of gestation, and some can affect the first trimester (BonDurant, 2007). This kind of losses is excluded from this thesis.

Non-infectious causes are often multifactorial and diagnosing a specific cause may be difficult, especially when working at population level, as is usual when studying domestic animals. In a classic review on prenatal loss in mammals, Wilmut *et al*. (1986) described three routes leading to prenatal death. Firstly, embryos may be abnormal because of inherited defects, errors at meiosis or fertilization or as a result of an environmental factor having a direct effect on the embryo. The maternal environment may be inadequate for supporting a normal pregnancy either because of abnormalities in the reproductive tract or as a result of an inappropriate hormone pattern. Finally, even with embryos and maternal environment inherently normal, embryos may die because they are not at the correct stage of development for the particular uterine environment. Nevertheless, some factors may act through more than one route, and asynchrony between conceptus and dam is probably involved only in losses before maternal recognition of pregnancy. In any case, although sometimes specific cause of pregnancy loss cannot be determined, identifying observable and measurable factors it is also of interest. Further elucidation of the factors associated to pregnancy loss may enable the development of more effective management regimens for efficient herd reproduction. It may also be the first step to determine the underlying physiological mechanisms leading to pregnancy loss. Several environmental-, nutritional-, management- and cow-related factors of a non-infectious nature have been already described to affect early foetal loss (Mares *et al*., 1961; Vanroose *et al*., 2000; López-Gatius *et al*., 2002; Silke *et al*., 2002; Chebel *et al*., 2004; Santos *et al*., 2004; Starbuck *et al*., 2004; Jousan *et al*., 2005; Grimard *et al*., 2006; BonDurant, 2007; Lee and Kim, 2007; Gabor *et al*., 2008; Santos *et al*., 2009; Silva-Del-Rio *et al*., 2009).

1.3 Endocrine changes in high producing dairy cows

It has been hypothesized that changes in reproductive physiology noted in cows under highly intensive systems could be due to a high rate of steroid metabolism (Wiltbank *et al*., 2006). This rationale arises from the fact that the higher dry matter intake of high producing dairy cows results in higher blood flow to the digestive tract, which in turn increases blood flow to the liver and enhances steroid metabolism (Rabiee *et al*., 2001; Rabiee *et al*., 2002; Sangsritavong *et al*., 2002; Vasconcelos *et al*., 2003). It would in fact not only affect oestrous expression and fertility, but also early foetal loss, a period when placental and foetal development are competing with a major drain of lactation. Accordingly, milk production can be negatively correlated to plasma progesterone concentrations at the onset of the foetal period (Rhinehart *et al*., 2009). Hence, it seems reasonable to suppose that one of the causes of increased risk of early foetal loss in high producing dairy cows could be sub-optimal concentrations of progesterone, either due to increased progesterone catabolism and sub-luteal function, or to both these factors, weakening establishment of early pregnancy.

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2

Conceptual and Methodological Frameworks

2 CONCEPTUAL AND METHODOLOGICAL FRAMEWORKS

Research performed for this thesis can be included in the field of veterinary medicine, considering late embryonic/early foetal loss the "disease" to study. Studies were performed in commercial dairy herds. Thus, the main approach has been on-farm surveillance of cows with special attention on the late embryonic and early foetal period through monitoring tools. In the area of reproductive biology of large animals, Ginther (1986) stated that: "*Gray-scale, real-time ultrasonography is the most profound technological advance in the field of animal research and clinical reproduction since the introduction of transrectal palpation and radioimmunoassay of circulating hormones*". This statement summarizes our main research tools: ultrasonography and evaluation of circulating hormones.

2.1 *The epidemiological approach*

Epidemiology is the study of disease in populations and of factors that determine its occurrence (Thrusfield, 2007). Although etymologically the word "epidemiology" refers to people (the Greek term "*demos*"), it generally refers to all animal populations, human and otherwise. An historical analysis of veterinary medicine, according to Kuhn's ideas of scientific development (Kuhn, 1962), has identified the epidemiological approach as the last *scientific revolution* in this discipline (Schwabe, 1982). Schwabe (1982; 1994) described the evolution of the science of animal disease management, depicting different phases. Each phase was characterized by a *disciplinary matrix* which was recognized and accepted by veterinary researchers and practitioners of that period. These *matrices* generated model approaches to solutions of the problems the field then recognized, what Kuhn called *paradigm*. Previous *paradigm* of veterinary medicine was initiated by the Microbiological revolution of the late 19th century where specific etiologic agents where considered "necessary and sufficient" for a specific disease. This *paradigm* enjoyed great scientific and public popularity, and undoubtedly gave great scientific strides in understanding diseases. However, in the mid 20th century some *anomalies* were detected, such as the recognition of "problem herds" unaffected by disease eradication campaigns, identification of complex diseases as well as non-host specificity of some infectious agents and the recognition of predisposing causes. All these facts led the paradigm to a *crisis* with a gradual recognition that a more holistic approach was needed for the identification, quantification and intensive examination of multiple, directly or indirectly causal, and often interacting disease determinants. The Epidemiological Revolution began and the current paradigm of "Surveillance and Selective actions" was established (Schwabe, 1982).

Epidemiological approaches, including mainly descriptive and analytical epidemiology, provide the framework where the present thesis is developed. Descriptive epidemiology includes observing and recording diseases and possible causal factors; analytical epidemiology consists in the analysis of observations using suitable diagnostic and statistical procedures (Thrusfield, 2007). Reproductive failure, in general, and non-infectious early foetal loss in particular, are multifactorial "diseases" difficult to assess with previous paradigms of disease management. Thus, the epidemiological approach permitted to define and quantify the clinical picture, which is fundamental in understanding the nature of the problem, in developing strategies for intervention, and in monitoring the outcome of a control program (Thurmond *et al.*, 1990).

Knowledge of factors associated with poor foetal survival can be used to direct management and herd health practices that minimize foetal loss and that improve predictability of abortion. Observing and analysing management-, environmental- and cow-related factors through logistic regression has been the main statistical procedure used to study early foetal loss. Logistic regression procedures allow analysing possible effects of numerous factors affecting a dependent dichotomic variable (Hosmer and Lemeshow, 1989), as is early foetal loss.

2.2 *Monitoring pregnancy*

Additionally to management-, environmental- and cow- related data, close conceptus surveillance during late embryonic period and early foetal period provided fundamental data to study early foetal loss.

The great advances in science usually result from new tools rather than from new doctrines (Dyson, 2006). The development and application of transrectal real-time ultrasonography to the study of bovine reproduction represents a technological breakthrough that has revolutionized knowledge of reproductive biology. New research information generated through ultrasonic imaging has clarified the nature complex reproductive processes in cattle including ovarian follicular dynamics, corpus luteum function, and conceptus development (Griffin and Ginther, 1992; King, 2006).

Probably, the main practical application of the clinical use of ultrasound in bovine practice is early pregnancy diagnosis, a fundamental feature for efficient animal production systems (Kahn, 1992). Together with pregnancy diagnosis, real-time visualisation of early conceptus has permitted an accurate method for studying late embryonic and early foetal periods and losses at these periods. Classical approaches for studying pregnancy loss in cattle include determining inter-oestrous intervals, attempting recovery of the conceptus, and different approximations for monitoring pregnancy. Using oestrus as a sign of non-pregnancy is a very imprecise method, mainly because of the difficulty of knowing when a cow is in oestrus (Roelofs *et al.*, 2010). Recovery of the conceptus and slaughtering methods can be used as a method for studying very early embryo period, but is by no means practical and economically viable for studying more advanced stages of pregnancy.

Many techniques have been employed over the years for monitoring pregnancy. Basically, these methods can be divided in biochemical monitoring and clinical examination. Measurement of progesterone concentrations in blood or milk samples has been a classical approach for diagnosing and monitoring pregnancy (Humblot *et al*., 1988; Humblot, 2001). However it monitors basically corpus luteum function, the main source of progesterone, and not a live embryo. Given that progesterone is necessary for pregnancy, measurement of progesterone is nearly 100 % accurate in identifying nonpregnant cows, however it is only from 75% to 85% accurate in correctly identifying pregnant cows and certainly it is not useful for detecting the presence of a live conceptus in utero. Other conceptus derived products have been proposed for monitoring pregnancy status. An "early pregnancy factor" was postulated, but later it was found inconsistently linked to pregnancy (Morton *et al*., 1987; Cordoba *et al*., 2001; Gandy *et al*., 2001). Placental derived substances such as bovine placental lactogens (detectable from about 60 days) or oestrone sulphate (expressed from about 70 days) can provide some information on placental status, but they are not suitable for early pregnancy monitoring (Byatt *et al*., 1987; Wallace, 1993; Patel *et al*., 1996; Holland *et al*., 1997; Hossner *et al*., 1997). On the contrary, pregnancy-associated glycoproteins (PAG), which constitute a large family of placenta-expressed proteins by the binucleate cells of the trophoblast, have been successfully used for pregnancy diagnosis and for determining the well being of the feto-placental unit (Skinner *et al*., 1996; Szenci *et al*. 1998a,b; Sousa *et al*., 2008).

The main method of clinical examination of bovine pregnancy is palpation per rectum. It has been and still is widely used, providing useful information. Skilled palpators are able to detect pregnancy in cattle as early as day 35 (Youngquist and Threlfall, 2006; Romano *et al*., 2007) and information on ovarian structures can be also obtained. However "hand-visualisation" can lead to some degree of error. With the advent of ultrasonography a continuation of the clinical examination is possible (Kofler and Hittmair, 2006), and reproductive tract can be visualised on images, providing much more accurate information on the embryo, its number, and ovarian structures. As stated before, ultrasonography has revolutionized our understanding of early pregnancy, and it is one of the methods that contribute more to enlighten the "black box" of early pregnancy (Macklon *et al*., 2002).

Close monitoring of the late embryonic and early foetal period mainly through ultrasonography and additionally with peripheral progesterone and PAG measurements provides therefore the basis for obtaining data referring to the status of gestation.

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3

Main Objectives

3 MAIN OBJECTIVES

The main aim of this thesis was to analyse, under an epidemiological framework, factors affecting pregnancy loss of non-infectious cause during the late embryonic and early foetal period in high-producing dairy cows. Development of therapeutic strategies for intervention was an additional objective. The main hypothesis for the therapeutic approach was that increasing rates registered in high producing dairy cows may be partly related to sub-optimal levels of progesterone. These general objectives were split in five specific objectives, as follows:

1) To analyse the effects of management- and cow-related factors of non-infectious nature affecting late embryonic and early foetal loss in North-eastern Spain high-producing dairy herds, summarizing previous studies in our area.

2) To determine whether certain management- or cow-related factors could affect progesterone concentrations on day 42 of gestation in high producing dairy cows.

3) To determine some pregnancy patterns during the early foetal period such as plasma progesterone concentrations, early foetal loss and corpora lutea formation in dairy cows treated with GnRH or Progesterone at the time of pregnancy diagnosis.

4) To investigate the effects of two progesterone-based oestrus synchronization treatments given on 51-57 days postpartum on early oestrous rate, early and cumulative conception rates and early foetal losses.

5) Based on the fact that low plasma concentrations of progesterone have been linked to foetal loss, the last main objective was to discuss recent therapeutic approaches designed to reduce the incidence of early foetal loss.

4

Factors of Non-Infectious Nature Affecting Late Embryonic and Early Foetal Loss in High Producing Dairy Herds in North-Eastern Spain

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4 Factors Of Non-Infectious Nature Affecting Late Embryonic and Early Foetal Loss in High Producing Dairy Herds in North-Eastern Spain

Abstract

Following a positive pregnancy diagnosis, late embryonic and early foetal loss is becoming the most common complication of gestation in high producing dairy herds in north-eastern Spain. The authors present the data published during the period 1996 to 2008, analysing the effects of factors of non-infectious nature affecting late embryonic and early foetal loss in high producing dairy herds. The results included a total of 15,525 pregnancies in well-managed, commercial, Holstein-Friesian high producing dairy herds over the period 1987 to 2007. Since the effects of different factors practically did not vary throughout the years, data from the different studies were pooled and the relative risk of every individual factor on foetal loss is described. Strong risk factors for pregnancy loss were parity (lactating cows versus heifers), the semen-providing bull, warm season and twin pregnancies, whereas the presence of an additional corpus luteum was identified as a strong positive factor favouring the maintenance of gestation. Progesterone and GnRH treatment had the potential to reduce the incidence of pregnancy loss in cows with one or more corpora lutea, respectively, in herds with high incidence of early foetal loss of non-infectious nature. From a practical point of view, assessment of normal development of gestation on days 60 and 90 after insemination is suggested.

Keywords: Early Foetal Loss, Milk production, Dairy cows

4.1 *Introduction*

In mammals, the successful course of gestation requires complex conceptus-maternal interactions within the microenvironments of the female genital tract. These interactions include processes, such as modulation of ovarian function, maternal recognition of pregnancy, implantation and placentation, very sensitive to disruption by several stresses. In the cow, placentation finishes before 60th day of gestation, period in which pregnancy is considered to be firmly established and the chances of loss are reduced (Ball, 1997); (Figures 4.1 – 4.4). The embryonic period of gestation extends from conception until the end of the differentiation stage (about 42 days), and the foetal period spans from 42nd day of gestation to parturition (Committee on Bovine Reproductive Nomenclature, 1972). In dairy cattle, although most pregnancy losses occur during the early embryonic

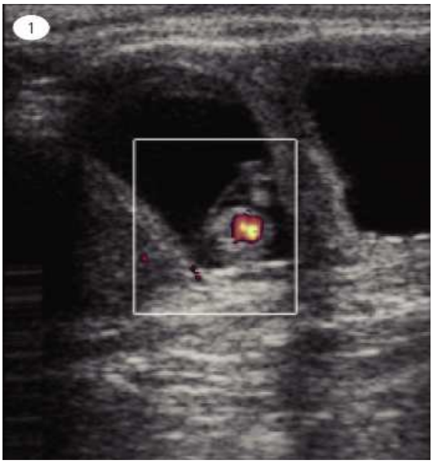

Fig. 4.1. Ultrasonographic picture of an embryo on 31st day after AI. Note the heart flow by using colour Doppler

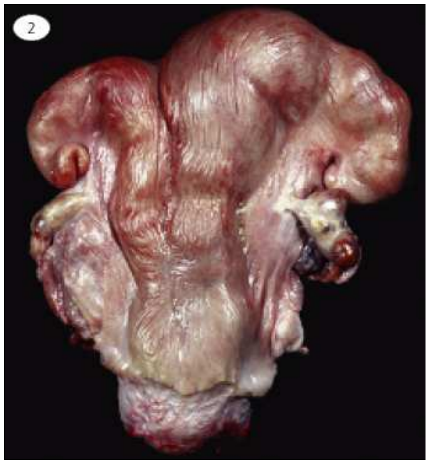

Fig. 4.2. Pregnant uterus on 39th day after AI

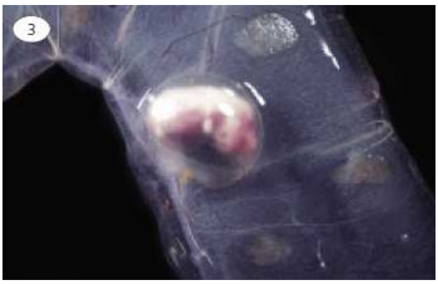

Fig. 4.3. Embryo within the membranes on 39th day after AI

Fig. 4.4. Embryo within the amnion on 39th day after AI

period (Peters, 1996), the risk of late embryonic and early foetal loss appears to increase under conditions of intensive management (Forar *et al*., 1995; Hanzen *et al*., 1999). Late embryonic and early foetal loss often escapes clinical diagnosis, and these pregnancy losses are assumed to be of a multi-factorial origin (Vanroose *et al*., 2000). Late embryonic and early foetal loss rate of 10% is a commonly accepted figure (Thurmond *et al*., 1990; Vanroose *et al*., 2000). In our geographical area (Labèrnia *et al*., 1996; López-Gatius *et al*., 1996; López-Gatius *et al*., 2002), and elsewhere (Gabor *et al*., 2008; Grimard *et al*., 2006; Jousan *et al*., 2005), management- and cow related factors of non-infectious nature have been described to affect late embryonic and early foetal loss in high producing dairy cows. Moreover, in warm countries, such as Spain, summer heat stress is a major factor affecting dramatically not only fertility (García-Ispierto *et al*., 2007; Labernia *et al*., 1998; López-Gatius, 2003; López-Gatius *et al*., 2005b) but also pregnancy loss (Garcia-Ispierto *et al*., 2006; López-Gatius *et al*., 2004a-c). In fact, late embryonic and early foetal loss are becoming the most common complication of gestation in high producing dairy cows in our geographical area, where more than 90% of pregnancy losses following pregnancy diagnosis occur usually before 90th day of gestation (López-Gatius *et al.*, 2002; López-Gatius *et al.*, 2004b); (Figures 4.5, 4.6). This brief presentation, based on our results in north-eastern Spain, expresses our views on factors affecting late embryonic and early foetal loss in high producing dairy herds and is by no means a review of the subject. A therapeutic approach is also proposed.

Fig. 4.5. Ultrasonographic picture of a dead embryo on 31st day after AI. Note the detachment of membranes.

Fig. 4.6. Ultrasonographic picture of a dead embryo on 33rd day after AI. Note the turbid amnion

4.2 *Selection of studies and data organization*

We selected 14 studies published in peer-reviewed journals during the period 1996 to 2008 analysing the effects of factors of non-infectious nature affecting late embryonic and early foetal loss in high producing dairy herds. Data derived from a reproductive control program conducted by the University of Lleida in north-eastern Spain, a geographical area under warm (May to September) and cool (October to April) conditions. Results included a total of 15525 pregnancies in well-managed, commercial, Holstein-Friesian high producing dairy herds over the period of 1987 to 2007. Logically, the concept of high milk production was different in 1987 (approximately 7500 kg/cow/year) compared to the current milk production in 2007 (slightly exceeding 12000 kg/cow/year in 2007). All pregnancy diagnoses were performed by the first author by palpation per rectum or by ultrasound.

Table 4.1 shows the factors affecting late embryonic and early foetal loss: risk factors, with no effect and preventive effect. Since the effects of different factors

Table 4.1. Factors affecting late embryonic and early foetal loss in high producing dairy herds. Data derived from compilation of several studies performed in north-eastern Spain.

Factors	Class	Pregnancy loss		Odds ratio (ranges)	Number of pregnancies (references[a])
		n	%		
Risk factors					
Parity	Heifers	47/1801	2.6		
	Cows	469/5184	9.0	3.6 - 3.7	6985 [1,2]
Bull	19 classes		2.5-21.2	2.1 - 3.4	2043[3,4]
Pospartum Body Condition Loss	Continuous			2.4	601[3]
Retained Placenta	Absence	249/2830	8.8		
	Presence	42/192	21.9	1.8	3022[5]
Previous Pyometra	Absence	280/2982	9.4		
	Presence	11/40	27.5	2.6	3022[5]
Season	Cool (October-April)	165/3894	4.2		
	Warm (May-September)	385/3009	12.8	1.6 - 5.4	6903[2,4,6,7,8]
Max THI[b] during gestation days 21 – 30	Continuous			1.05	1391[7]
Embryo number	Singletons	239/3394	7.0		
	Twins	153/624	24.5	3.1 – 6.9	4018[4,7,9,10]
Factors with no effect					
Previous ovarian cysts	Absence	340/3458	9.8		
	Presence	15/165	9.1		3623[3,5]
Milk production	< 40 kg	253/2810	9.0		
	≥ 40 kg	153/1722	8.9		4532[3,4,7,8]
Days in milk	< 90 days	121/1450	8.3		
	≥ 90 days	285/3082	9.2		4532[3,4,7,8]
Preventive factors					
Additional corpus luteum	Absence	360/3643	9.9		
	Presence	6/363	1.7	0.12 - 0.32	4006[3,4,6,7,11]
Progesterone supplementation (Days 36 – 42)	Absence	66/549	12		
	Presence	29/549	5.3	0.41	1098[8]

[a] References: [1]Labèrnia *et al*. 1996; [2]López-Gatius *et al*. 2004c; [3]López-Gatius *et al*. 2002; [4]López-Gatius *et al*. 2004b; [5]López-Gatius *et al*. 1996; [6]Bech-Sàbat *et al*. 2008; [7]García-Ispierto *et al*. 2006; [8]López-Gatius *et al*. 2004a; [9]López-Gatius and Hunter 2005; [10]López-Gatius *et al*. 2005a; [11]López-Gatius *et al*. 2006

[b] Max THI: maximal temperature-humidity index

practically did not vary throughout the years, data from the different studies were pooled and presented as proportions and ranges for the relative risk of every individual factor on foetal loss. If it is not indicated by including references, all data derive from Table 4.1.

4.3 *Risk factors*

4.3.1 Parity

The influence of parity status (non-lactating heifer versus lactating cow) clearly affects the proportion of pregnancy losses. On a total of 1801 pregnant heifers, 2.6% suffered foetal loss, whereas 9% losses were registered for 5124 lactating pregnant cows (compilation of two studies). Our values are comparable to those obtained between 1949 and 1955: 2.5% of losses for heifers and 13% for multiparous Holstein-Friesian cows (Mares *et al*., 1961), and to those compiled from the literature by Santos *et al*. (2004a): pregnancy loss was 10.7% for lactating cows (compilation of 10 studies) and 4.2% for dairy heifers (compilation of five studies). These findings suggest that genetic selection for high milk production did not affect the incidence of losses during the last decades. Parturition seems to have mild effect for the development of a subsequent gestation (Ball, 1978; Thurmond *et al*., 1990). Metabolic stress associated with lactation could also compromise foetal survival.

4.3.2 The bull

Cows inseminated by a particular bull had from 2.1 to 3.4 times higher risk of pregnancy loss than the remaining cows, on a total of 2043 pregnancies (compilation of two studies). Proportions ranged from 2.5% to 21.2% losses for the different bulls. In a further study, where analyses were performed on a small study population (98 pregnant cows), from the 10 inseminating bulls included in the study, one bull was related to increased foetal loss by odds ratio of 21.7, whereas another bull was attributed foetal loss rate, reduced by odds ratio of 0.08 (López-Gatius *et al*., 2007b). Due to the exceptional nature of these latter findings within a reduced study population, the data are not included in Table 4.1. On the other hand, in several studies including 2489 further pregnancies, we could not find a bull effect (Garcia-Ispierto *et al*., 2006; López-Gatius *et al*., 2004a).

Pregnancy loss risks have been linked to specific bulls by Markusfeld-Nir (1997) in an epidemiological study on 58048 pregnancies. The incidence of pregnancy loss between 30 and 80 days after AI by using semen from a single bull has been reported up to 44% (Bulman, 1979) and, more recently, up to 39% (Pegorer *et al*., 2007). Possible reasons for the bull effect include anatomical and genetic defects (Ball, 1997), and infection caused by the use of contaminated semen should not be excluded. Although primary attention is usually directed towards bull effects on fertility, once a cow is pregnant the semen providing bull seems to emerge as an important risk factor for early foetal loss.

Ruminant placenta synthesizes substantial amounts of glycoproteins related to pregnancy (PAGs), including the PAG-1 and PAG-2 subgroups. Although their physiological function remains unknown, plasma concentrations of PAG-1 subgroup have been used both for pregnancy diagnosis and as a marker of foetal/placental viability (Skinner *et al.*, 1996; Szenci *et al.*, 1998a,b), or to monitor pregnancy failure during the late embryo and early foetal period (Humblot, 2000; López-Gatius *et al.*, 2007b; Mialon *et al.*, 1993; Szenci *et al.*, 2000; Taverne *et al.*, 2002). A substantial variation in the PAG-1 level was accounted for the individual bull, with mean PAG-1 values ranging from 1.6 to 4.5 ng/ml for the different bulls (López-Gatius *et al.*, 2007b). These differences proved to be very significant in a further study (López-Gatius *et al.*, 2007a). Although we could not find significant interactions when analyzing factors affecting foetal loss in these studies, it is reasonable to expect a significant paired paternal-PAG level interaction effect in more extensive studies. For example, bulls linked to pregnancies with very low or very high PAG-1 levels could be associated with a higher risk of foetal loss. If this is true, PAG-1 measurement should be a useful tool to detect bulls favouring foetal loss. Anyway, artificial insemination centres could have a key role in including foetal loss as a possible genetic trait in the sire-selection programs.

4.3.3 Previous postpartum disorders

Cows showing a 1-unit drop in body condition score from parturition to 30 days postpartum had a risk of foetal loss 2.4 times higher than cows that maintained their body condition. Pregnancy loss rates were 2.6 and 1.8 times higher in cows with previous pyometra and retained placenta, respectively, than in cows without these disorders (López-Gatius *et al.*, 1996). Previous retained placenta or pyometra could have caused alterations in the uterine environment that resulted in subsequent pregnancy loss. Injuries to the uterine mucosa could impair nutrition and attachment of the conceptus during the implantational process. However, it is more difficult to explain why excessive loss of body condition during early lactation is related to subsequent pregnancy loss. Probably, an abrupt loss of nutritional status postpartum impairs uterine involution and causes pregnancy failure when the placentomes develop in the early foetal period. It is interesting to note that irrespective of the calving-gestation interval all cows suffering from previous postpartum disorders, either metabolic or uterine, had a higher risk of pregnancy loss than the others without the disorder. These findings suggest a long time effect for postpartum stresses.

4.3.4 Clinical diseases

The link between mastitis and embryonic mortality has been proposed as one example of how the interface between the immune and reproductive systems can impinge on reproductive success (Hansen *et al.*, 2004). Furthermore, the inflammatory processes implicated in acute mastitis or lameness can elevate prostaglandin secretion, cause luteolysis and have been related to foetal loss (Graham *et al*., 1995). We analysed the possible effect of clinical diseases on late embryonic and early foetal loss only in one single study (López-Gatius *et al*., 2002). Because of its low incidence (a total of 9.3% of 601 pregnancies), acute mastitis and lameness were grouped as one single factor of clinical disease (data not included in Table 4.1). Logistic regression analysis indicated that there was no significant effect of clinical disease on foetal loss. The use of flunixin meglumine, a non-steroid anti-inflammatory drug that inhibits prostaglandin biosynthesis (Kindahl *et al*., 1999), for the treatment of clinical disease in our study could overcome negative effects of mastitis and lameness on pregnancy retention. In fact, clinical mastitis (Chebel *et al*., 2004; McDougall *et al*., 2005; Santos *et al*., 2004b; Schrick *et al*., 2001) and elevated somatic cell counts (Jousan *et al*., 2005; Moore *et al*., 2005) have been related to foetal loss.

4.3.5 Season

In five studies, including 6903 pregnancies, the risk of pregnancy loss was found to be from 1.6 to 5.4 times higher in cows becoming pregnant in the warm (May to September) than in the cool (October to April) period of the year. The highest proportion of losses during the warm period (54%) was registered in twin pregnancies (López-Gatius *et al*., 2004b). Furthermore, a hard relationship between heat stress during the peri-implantation period and subsequent late embryonic and early foetal loss was also registered. The likelihood of pregnancy loss increased by a factor of 1.05 for each additional unit of the mean maximum temperature-humidity index from the 21st to 30th days of gestation. Several factors can explain losses under heat stress, either centred in lesions of the conceptus or in the corpus luteum function. However, cow-environment interactions are difficult to evaluate. We have now two reasons to try to better understand the effect of climatic stress on late embryonic and early foetal loss. Firstly, the fact that introduction in the herds of fans and water sprinklers for the warm season in the late nineties was associated with a substantial decrease in the foetal loss rate (unpublished data). And secondly, that the climatic variables in the referred studies were always monitored in a meteorological station located several km-s away from the herds and the data obtained were only derived from mean daily values of temperature and relative humidity. Analyses of possible relationships between real farm climate conditions (probes in the cubicles area), including their evolution during the day (measurements every 30 min.), and pregnancy maintenance are the subject of our next work.

4.3.6 Twin pregnancies

Probably twin pregnancies are the main factors related to late embryonic and early foetal loss in high producing dairy herds. Using cows carrying singletons as reference, the minimum likelihood of pregnancy loss for twin pregnancies was a factor 3.1 (compilation of five studies including 4018 pregnancies). As noted above, in the warm period up to 54% of losses were registered in twin pregnancies (López-Gatius *et al*., 2004b).

The twinning rate has increased alongside milk production over the last two decades (Kinsel *et al*., 1998; Nielen *et al*., 1989). The increase in the twinning rate, exceeding up to 9%, have been linked to increased milk production in an epidemiological study on 52362 lactations (Kinsel *et al*., 1998). In high producers, the rate of double ovulation may be over 20% (Fricke and Wiltbank, 1999) and 25% for cows in their third or more lactation period (López-Gatius *et al*., 2005a). Genetics appears to be a major regulatory factor for twinning rates. In an analysis of data related to 1324678 births in 37174 sires, it was shown that sires born after 1990 had a higher incidence of twins than sires born before 1980 (Johanson *et al*., 2001). It is reasonable to suggest that increased twinning is a consequence of selection for milk yield, but aside from genetic progress, improvements in nutrition and management practices have led to a continuous increase in the milk yield. Probably, the need to improve management at the farm level has diminished the risk of embryo loss in twin pregnancies and thus raised the twinning rate. It is therefore foreseeable that over the years to come, the twinning rate will continue to increase along with milk production.

Twin pregnancies are undesirable in dairy cattle since they increase not only the risk of early pregnancy loss, but also have many negative effects, such as increased abortion, dystocia, retained placenta, calf mortality, occurrence of freemartins, postpartum therapy, and longer rebreeding intervals (Nielen *et al*., 1989). The profitability of the herd diminishes drastically as frequency of twin births increases (Eddy *et al*., 1991). From a national perspective in the USA, costs related to twinning were around $55 million per year (Johanson *et al*., 2001). The increase in twinning rate has been also observed during summer months (De Rensis and Scaramuzzi, 2003), increasing this way their negative effects in geographical areas under heat stress conditions. Such negative effects of twinning might be diminished by reducing the embryo number in dairy cows.

Embryo reduction methods are used in assisted reproduction in humans (Mansour *et al*., 1999) and in the treatment of twin pregnancies in mares (Macpherson and Reimer, 2000). Good success (75%) was reported with manual crushing of a twin if performed before 30 days of gestation in the mare (Pascoe *et al*., 1987). We evaluated manual rupture of the amniotic vesicle of a twin embryo as an approach to the problem (López-Gatius, 2005). Our results indicated that amnion rupture without further treatment resulted in pregnancy loss in 100% of cases (11/11), whereas the procedure of rupturing the amnion plus progesterone supplementation may provide a satisfactory way for twin reduction in dairy cattle: 4 of 11 animals retained gestation. However, in one of these latter four surviving pregnancies, one embryo survived amnion rupture and the cow bearing it had twins at parturition. Transvaginal ultrasound-guided embryo aspiration could be an alternative to amnion rupture for reduction of twin pregnancy in the cow, as it has been also proposed in the mare (Bracher *et al*., 1993; Mari *et al*., 2004) and in humans (Coffler *et al*., 1999).

The development of 211 twin pregnancies was monitored in order to determine the best time for an embryo reduction approach (López-Gatius and Hunter, 2005). Pregnancy was diagnosed by ultrasound between 36 and 42 days after insemination. Animals were then subjected to weekly ultrasound examinations until 90th day of gestation or until pregnancy loss. Embryo death was registered in one of the two embryos in 35 cows (16.6%), 33 of them at pregnancy diagnosis. Pregnancy loss occurred in 22 of these cows between 1 and 4 weeks later. Thus, 13 (37%) cows carrying one dead of the two embryos, maintained gestation. In a more recent study (data unpublished), a single embryo death with its living co-twin was recorded in 20% (98/491) of cows bearing twins, diagnosed pregnant between 28th and 34th days, with a subsequent pregnancy loss of 62%. Since, as noted above, progesterone supplementation was useful for artificial twin reduction, treatment was tried in 17 further twin pregnancies with one dead embryo (with no control cows) and losses were registered just in two cows. These findings suggest that the use of artificial reduction of embryo number in clinical practice could be attempted at pregnancy diagnosis, as early as possible, on twin pregnancies in which both embryos were detected to be viable. In cows carrying one dead of the two embryos, a therapeutic approach includes progesterone supplementation at pregnancy diagnosis.

4.4 *Factors with no effect*

Previous ovarian cysts (compilation of two studies with 3623 pregnancies) and milk production at pregnancy diagnosis and days open at gestation (compilation of four studies with 4532 pregnancies) did not affect the pregnancy loss rate. In most of these studies, only healthy cows, and improved environmental factors necessary for high production, such as better nutrition, housing, health and management could explain why these variables, affecting often other reproductive parameters, had not any effect on pregnancy loss. Concerning milk production, our findings agree with several studies where there is little or no indication that milk production is a risk factor for increased pregnancy losses in dairy cattle (Chebel *et al*., 2004; Jousan *et al*., 2005; McDougall *et al*., 2005; Santos *et al*., 2004a).

4.5 *Preventive factors*

4.5.1. Additional corpus luteum

Defined as pregnancies with a number of corpora lutea higher than the number of embryos, additional corpus luteum has demonstrated to be a very strong factor favouring pregnancy maintenance. On a total of 363 pregnant cows with an additional corpus luteum, 1.7% suffered foetal loss, whereas 9.9% losses were registered for 3643 pregnant animals with no additional corpus luteum. Odds ratios of losses were from 0.12 to 0.32 for cows with additional corpus luteum, related to cows without it (compilation of five studies including 4006 pregnancies). In three of the five studies in which a total of 126 cows, carrying singletons with two corpora lutea were registered, no cow suffered pregnancy loss (Bech-Sàbat *et al.*, 2008; López-Gatius *et al.*, 2002; López-Gatius *et al.*, 2006).

Progesterone is unequivocally required for supporting gestation (Spencer *et al.*, 2004), pregnancy maintenance has been positively correlated to plasma concentrations of progesterone on the 5th week of gestation (Starbuck *et al.*, 2004), and progesterone concentrations influence secretory functions of trophoblasts and pituitary during the first trimester of gestation (Ayad *et al.*, 2007). However, one of the consequences of high milk production is increased metabolic rate linked to a greater dry matter intake. This process reduces plasma concentrations of steroid hormones, such as progesterone (Sangsritavong *et al.*, 2002), with obvious impacts not only on fertility but also on gestation. In effect, milk production can affect negatively plasma progesterone concentrations at the onset of the foetal period (Bech-Sàbat *et al.*, 2008). Therefore, it seems reasonable to suppose that one of the causes of late embryonic and early foetal loss in high producing dairy cows could be the suboptimal concentrations of progesterone, either due to the increased progesterone catabolism, the sub-luteal function, or both. Thus, strategies that induce the formation of an additional corpus luteum may help to increase progesterone concentrations in high producing dairy cows. For example, treatment with GnRH at AI and 12 days later increased the likelihood of the production of additional corpus luteum by a factor of 3.7 (López-Gatius *et al.*, 2006), and induction by GnRH treatment of an accessory corpus luteum on 27th day of gestation favoured pregnancy retention between the 45th and 90th (Bartolome *et al.*, 2006). On the other hand, identification of methods to decrease excessive hepatic clearance without compromising dry matter intake should not be discarded. Some dietary ingredients seem to reduce progesterone catabolism (Lemley *et al.*, 2008).

4.5.2 Progesterone supplementation

In order to test the hypothesis that sub-optimal progesterone concentrations may compromise conceptus development, we supplemented pregnant cows with exogenous progesterone (López-Gatius *et al.*, 2004a). Treatment were fitted at pregnancy diagnosis, between 36th and 42th day post-insemination, with a progesterone releasing intra-vaginal device (PRID) containing 1.55 g of progesterone, for 28 days. Based on the odds ratio, the risk of pregnancy loss was 2.4 times higher in non-treated cows than in treated ones: 12% (66/549) versus 5.3% (29/549) of losses, respectively. In a more recent study where treatment was fitted on 28th day of gestation, results were similar: 16% (16/97) losses for non-treated and 6% (6/102) losses for treated cows (Bech-Sabat *et al.*, 2007). These results support the hypothesis that sub-optimal progesterone concentrations in high producing dairy cows may compromise conceptus development. Under these conditions, intra-vaginal progesterone supplementation has the potential to reduce the incidence of pregnancy loss during the early foetal period.

4.6 *Anomalous pregnancies*

Within a large-scale ultrasound pregnancy diagnosis programme, a very low incidence (0.5%: 16/3094) of anomalous pregnancies was recorded (Serrano *et al*., 2009). The following anomalies were detected on 35-41st days of gestation in cows carrying singletons with one single corpus luteum: embryo death in eight cows (0.3%); the embryo placed in the uterine horn contralateral to the corpus luteum in seven cows (0.2%); and pyometra in a single case (0.03%). All these animals suffered pregnancy loss during the early foetal period. This very low incidence of anomalous pregnancies suggest that, in the case of high producing dairy herds with good management practices, pregnancies subsequent to cases of fertilisation failure or with abnormal embryo development fail during the early embryonic period, from 12 to 16 days post-insemination, which is the time when most pregnancy losses occur in the cow (Hanzen *et al*., 1999; Vanroose *et al*., 2000). However, two pregnancy loss episodes over three and two weeks, respectively, in which a high proportion of embryonic death at 28-34 days of gestation, were more recently registered (data unpublished). Of a total of 42 diagnosed pregnant cows carrying singletons, embryonic death was diagnosed in 15 animals (35.7%) and in two cows carrying twins (death of both embryos). In both episodes, events related to the disorder occurred in a too distant past to obtain accurate information about possible causes. Probably, the reason for losses was the bad quality of finishing silage stocks one-two weeks before losses. Feed and forage toxicants affect embryo survival and foetal development (McEvoy *et al*., 2001; Santos *et al*., 2004a).

4.7 *Timing of early foetal loss*

In a study on a total of 1442 lactating cows, diagnosed pregnant between 36 and 42 days after insemination, pregnancies were monitored weekly until 90th day of gestation or until pregnancy loss (López-Gatius *et al*., 2004b). The average day of pregnancy loss for cows with singletons (1310) was 52 days and ranged from 45 to 61 days. The average day of pregnancy loss for cows with twins (n=132) was 75 days and ranged from 46 to 90 days. 75% of the twin pregnancy losses were registered between 68 and 90 days of gestation. Grouping singleton and twin pregnancies, the average day of pregnancy loss was 58 days and 75% of the pregnancy losses were registered between 45 and 60 days of gestation. Our data showed that the early foetal loss period in singleton pregnancies occurs earlier than in twin pregnancies.

Our results are comparable to those from Santos *et al*. (2004a), who noted substantial losses until 42-46 days after insemination in a compilation of 10 studies.

It seems that most early foetal losses occur during the final process of placentation (40-50 days of gestation), a time when placental and foetal development are competing with the major drain of lactation. In the case of twin pregnancies, the period of losses can be prolonged, perhaps due to the fact that cows carrying twins with one dead of the two conceptuses can maintain gestation for several weeks before pregnancy loss (López-Gatius and Hunter, 2005).

Records of losses during the early foetal period are not new. Based on monthly tests of non-return rates, Kidder *et al*. in the fifties (Kidder *et al*., 1954) estimated that approximately 40% of pregnancies were lost by 60-90 days after insemination. The 60-90 days non-return percentage minus 5.5 was the estimate of the pregnancy rate.

4.8 A therapeutic approach

In a recent study (Bech-Sàbat *et al*., 2009), cows at pregnancy diagnosis were randomly assigned to progesterone (n=312) or GnRH (n=294) treatment groups. Logistic regression procedures revealed that in cows with a single corpus luteum, the probability of pregnancy loss between the first (28-34th days) and second (56-62th days) pregnancy diagnosis decreased by a factor of 0.51 in the progesterone group compared to the GnRH group. However, in cows with two or more corpora lutea, progesterone treatment increased the likelihood of pregnancy loss by a factor of three, compared to GnRH treatment. In cows carrying twins, the conceptus reduction rate was higher (P=0.02) for the GnRH (36%) than for the progesterone (16.4%) group. These results suggest that at early pregnancy diagnosis (28-34th days), it is very important to register the number of corpora lutea as the number of conceptuses. The practical implications of these findings are that in herds with high incidence of early foetal loss of non-infectious nature, treatment at the time of pregnancy diagnosis with progesterone in cows with one corpus luteum and with GnRH in cows with two or more corpora lutea should offer considerable benefits.

4.9 Concluding remarks

Once a cow has been diagnosed pregnant, late embryonic and early foetal loss is becoming the most common complication of pregnancy in high producing dairy cows. Factors strongly affecting late embryonic and early foetal loss are parity (lactating cows versus heifers), semen-providing bull, warm season and twin pregnancies, whereas the presence of an additional corpus luteum has been identified as a strong positive factor favouring the maintenance of gestation. Progesterone and GnRH treatment had the potential to reduce the incidence of pregnancy loss in cows with one or two or more corpora lutea, respectively, in herds with a high incidence of early foetal loss of a non-infectious nature. From a practical point of view, assessment of normal development of gestation on 60th and 90th days after insemination is suggested.

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5

Factors Affecting Plasma Progesterone in the Early Foetal Period in High Producing Dairy Cows

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5 FACTORS AFFECTING PLASMA PROGESTERONE IN THE EARLY FOETAL PERIOD IN HIGH PRODUCING DAIRY COWS

Abstract

The aim of the present study was to determine whether certain animal- or management related factors could affect plasma progesterone concentrations on Day 42 of gestation in high producing dairy cows. Factors affecting early fetal loss also were analyzed. The study population was comprised of 199 pregnant cows classified as having high (≥9 ng/ml) or low (<9 ng/ml) plasma progesterone concentrations. Through logistic regression procedures it was determined that, based on the odds ratio, cows with two or more corpora lutea were three times more likely to have high progesterone concentrations than cows with a single corpus luteum. Low producing cows during the warm season were 2.86 times more likely to have high progesterone concentrations than the remaining cows. Primiparous cows with high concentrations (>4 ng/ml) of pregnancy associated glycoprotein-1 (PAG-1) were 2.73 times more likely to have high progesterone concentrations than the remaining cows. Of the 199 pregnancies, 25 (12.6%) suffered early fetal loss: 22/136 (16.2%) during the warm season and 3/63 (4.8%) during the cool season, all in cows without additional corpora lutea. Based upon the odds ratio, cows without an additional corpus luteum were 3.67 times more likely to suffer fetal loss during the warm season than during the cool season. Our results indicate that milk production, the presence of two or more corpora lutea and plasma PAG-1 concentrations can affect plasma progesterone concentrations at the onset of the fetal period. The presence of an additional corpus luteum strongly diminished the risk of early fetal loss during the warm period.

Keywords: progesterone, early fetal period, milk production, PAG, dairy cows

5.1 Introduction

Conceptus-maternal interactions in mammals include implantation, modulation of ovarian function, maternal recognition of pregnancy and placentation. These processes are all so delicate that their disturbance can lead to the end of gestation. In dairy cattle, prenatal loss is probably one of the most important factors affecting reproductive performance, and consequently has a substantial impact on the profitability of cow production (Ball, 1997; Vanroose *et al*., 2000). The embryonic period of gestation extends from conception until the end of the differentiation stage (about 42 days), and the fetal period spans from Day 42 of gestation to parturition (Committee on Bovine Reproductive Nomenclature, 1972).

Although most pregnancy losses occur during the early embryonic period (Peters, 1996), the risk of early fetal loss appears to increase under conditions of intensive management (Forar *et al*., 1995; Hanzen *et al*., 1999). An early fetal loss rate of 10% is a commonly accepted figure (Ball, 1997; Thurmond *et al*., 1990; Vanroose *et al*., 2000) and is often of multifactorial origin and difficult to diagnose (Vanroose *et al*., 2000). In our geographical area (Labèrnia *et al*., 1996; López-Gatius *et al*., 2002) and elsewhere (Grimard *et al*., 2006; Santos *et al*., 2004; Silke *et al*., 2002), management- and cow related factors of a non-infectious nature have been described to affect early fetal loss in high producing dairy cows.

One of the consequences of high milk production is an increased metabolic rate linked to a greater intake of dry matter. This process reduces plasma concentrations of steroid hormones such as progesterone (Sangsritavong *et al*., 2002), with obvious impacts not only on fertility but also on gestation. Progesterone is unequivocally required for maternal support of conceptus survival and development (Spencer *et al*., 2004). In a previous study, we demonstrated that progesterone supplementation in high producing dairy cows during the early fetal period reduces pregnancy losses (López-Gatius *et al*., 2004a). We also identified the presence of an additional corpus luteum as a preventive factor for early fetal loss (Garcia-Ispierto *et al*., 2006; López-Gatius *et al*., 2002; López-Gatius *et al*., 2004b). Further, pregnancy maintenance has been positively correlated to plasma concentrations of progesterone on Week five of gestation (Starbuck *et al*., 2004). Thus, it seems reasonable to suppose that one of the causes of early fetal loss in high producing dairy cows could be suboptimal concentrations of progesterone. The literature lacks studies examining the factors affecting plasma progesterone concentrations during the early fetal period. Thus, the aim of the present study was to determine whether factors such as herd, lactation number, days in milk, season, number of corpora lutea, milk production and plasma pregnancy-associated glycoprotein-1 (PAG-1) concentrations could affect plasma progesterone concentrations on Day 42 of gestation in high producing dairy cows. Also we assessed the effects of cow and management variables previously found to be significantly correlated with early fetal loss in our geographical area.

5.2 *Materials and Methods*

5.2.1 Cattle and herd management

Our study was performed on two commercial Holstein-Friesian dairy herds in northeastern Spain, comprising a mean of 730 mature animals (160 and 570 per herd, respectively). Cows becoming pregnant from April 2004 to March 2006 were included in the study. Mean annual milk production was 11200 kg per cow. The cows, reared within the herds, calved all the year round and were milked three times per day and fed complete rations. Feeds consisted of cotton-seed hulls, barley, corn, soybean and bran, and roughage, primarily corn, barley or alfalfa silages and alfalfa hay. Rations were in line with NRC recommendations (National Research Council, 2001). All the animals were tested free of tuberculosis and brucellosis. All cows were bred by artificial insemination using semen from 28 independent bulls of proven fertility. The mean annual culling rate for the study period was 28%. The conception rate (number of pregnant cows as a percentage of the total number of AI) was 32% for the study period. The final study population comprised 199 pregnancies of the total 1358 pregnancies diagnosed during the study period. For each of the two cows which became pregnant during the warm period (with a higher risk for pregnancy loss), approximately one single cow becoming pregnant during the cool period was included in the study.

The mean lactation number of all the cows included in the study was 2.3 ± 1.4, ranging from one to eight lactations. Mean milk production was 37.1 ± 5.5 kg and 42.9 ± 8.4 kg for primiparous and multiparous cows, respectively.

5.2.2 Pregnancy diagnosis, number and viability of embryos and number of corpora lutea

Pregnancy was diagnosed by transrectal ultrasonography on Day 42 of gestation using a portable B-mode ultrasound scanner (Scanner 100 Vet equipped with a 5.0 MHz transducer; Pie Medical, Maastricht, The Netherlands). Scanning was performed along the dorsal/lateral surface of each horn. The viability of fetuses was determined by detecting the fetal heartbeat. The presence of twins was established through the observation of two fetuses in different positions within one uterine horn on two screen scans, two fetuses simultaneously present on screen, or one fetus in each uterine horn. Each ovary was also scanned to identify the number of corpora lutea. Only cows diagnosed with live fetuses were included in the study. Confirmation of pregnancy was performed by palpation per rectum on Days 60 and 90 of gestation. Fetal loss was registered when 60 day- or 90 day-diagnoses proved negative. All pregnancy diagnoses were performed by the same operator.

5.2.3 Blood sampling

Blood samples were withdrawn from the coccygeal vein of each pregnant animal immediately after the ultrasound exam into heparinized vacuum tubes (BD VacutainerTM, Becton, Dickenson and Company, Plymouth, UK). The samples were centrifuged (10 min at 1600 x g) within 30 min of collection, and the plasma stored at -20ºC until assayed.

5.2.4 Progesterone radioimmunoassay

Progesterone concentrations were determined in plasma using a direct method (without extraction) as previously described in detail (López-Gatius *et al.*, 2007a). The minimum detection limit of the P4-RIA technique used was 0.15 ng/ml, and intra-assay and inter-assay coefficients of variation were 5.1% and 10.5%, respectively.

5.2.5 PAG-1 radioimmunoassay

Pregnancy-associated glycoprotein-1 concentrations were determined in plasma using a double antibody radioimmunoassay procedure (RIA-706) (López-Gatius *et al.*, 2007a; Perenyi *et al.*, 2002a,b). Rabbit polyclonal antisera AS#706 was raised against caprine PAG55kDa+62kDa (accession numbers P80935 and P80933) preparation according to the Vaitukaitis method (Vaitukaitis *et* al., 1971). The minimum detection limit (MDL) for the RIA procedure was 0.26 ng/ml. Intra-assay and inter-assay CV were 3.1% and 10.3%, respectively.

5.2.6 Data collection and analysis

In the geographical area of study, there are only two clearly differentiated meteorological periods: warm (May to September) and cool (October to April) (Labernia *et al.*, 1998). Reproductive variables are generally significantly impaired in the warm period (López-Gatius, 2003). For this reason, pregnancy diagnosis dates were used to analyze the effect of the season (warm versus cool period) on progesterone concentrations.

The following data were recorded for each animal on pregnancy diagnosis: herd, parity (primiparous versus multiparous), service sire, milk production (average of the 3 days prior to pregnancy diagnosis), days in milk, number of fetuses, number of corpora lutea, season, plasma concentrations of PAG-1 and progesterone. The variables possibly affecting progesterone concentrations on pregnancy diagnosis and subsequent pregnancy losses are listed in Table 5.1. Although the effect of the sire on fetal loss has been demonstrated in previous studies (López-Gatius *et al.*, 2002; López-Gatius *et al.*, 2004b; Pegorer *et al.*, 2007; Starbuck *et al.*, 2004), this variable was excluded from the analyses due to the large number of bulls (28 independent bulls) used to inseminate the cows included in the study.

In both studies, logistic regression analyses were performed using the SPSS package, version 13.0 (SPSS Inc., Chicago, IL, USA) according to the method of 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 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 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.

Table 5.1. Risk factors assessed for their effects on high plasma progesterone (P4) concentrations (>9 ng/ml)[a] and early fetal loss[b] (N=199 pregnancies)

Risk factor	Mean ± S.D. (ranges)	Class description	N (%)[d]	High P4 N (%)[e]
Multiple corpus luteum[a]		One corpus luteum	143 (71.9)	35 (24.5)
		Two or more corpora lutea	56 (28.1)	26 (46.4)
Additional corpus luteum[b,c]		Absence	175 (87.9)	52 (29.7)
		Presence	24 (12.1)	9 (37.5)
Twin pregnancy [b]		Singletons	166 (83.4)	43 (25.9)
		Twins	33 (16.6)	18 (54.5)
Season[a,b]		Cool (October-April)	63 (31.7)	19 (30.2)
		Warm (May-September)	136 (68.3)	42 (30.9)
Parity [a,b]		Primiparous	71 (35.7)	21 (29.6)
		Multiparous	128 (64.3)	40 (31.3)
Milk production [a,b]	40.8 ± 8 (20-65)	< 40 kg	95 (47.7)	38 (40)
		≥ 40 kg	104 (52.3)	23 (22.1)
Plasma progesterone[b]	7.7 ± 3 (2.2-19.8)	< 9 ng/ml	138 (69.3)	
		≥ 9 ng/ml	61 (30.7)	
Herd[a,b]		1	49 (24.6)	12 (24.5)
		2	150 (75.4)	49 (32.7)
Plasma PAG-1[a,b]	3.3 ± 2.2 (0.2-19.1)	< 2.5 ng/ml	84 (42.2)	20 (23.8)
		2.5 – 4 ng/ml	63 (31.7)	17 (27)
		> 4 ng/ml	52 (26.1)	24 (46.2)
Days in milk[a,b]	201 ± 97 (63-727)	< 130 days in milk	44 (22.1)	19 (43.2)
		≥ 130 days in milk	155 (77.9)	42 (27.1)

[d]With respect to the total number of animals.
[e]With respect to the total number of animals in each class.
[c]Pregnancies with more corpora lutea than fetuses.

We continued modeling until all the main effects or interaction terms were significant according to the Wald statistic at P <0.05.

5.2.6.1 Progesterone concentrations

Cows were classified as having low (<9 ng/ml) or high (≥9 ng/ml) plasma progesterone concentrations (Ayad *et al.*, 2007). High progesterone was considered as the dependent variable, and multiple corpora lutea (defined as the presence of two or more corpora lutea), season (warm period), parity (multiparous), and high milk production (≥40 kg milk at pregnancy diagnosis) were considered dichotomous variables (where “1” denotes presence and “0” absence). PAG-1 concentrations, days in milk, and herd (class variables) were factors in the analysis. Three classes were established for PAG-1 concentrations: <2.5, 2.5-4 and >4 ng/ml (López-Gatius *et al.*, 2007b). Two classes were established for days in milk: early (<130 DIM) and late (≥130 DIM) lactation period.

5.2.6.2 Fetal loss

Fetal loss was considered as the dependent variable and the presence of an additional corpus luteum (defined as pregnancies with a number of corpora lutea higher than the number of fetuses), presence of twins, season (warm period), parity (multiparous), high progesterone concentration (≥9 ng/ml) and high milk production (≥40 kg milk at pregnancy diagnosis) were considered dichotomous variables. PAG-1 level, days in milk and herd (class variables) were factors in the analysis.

5.3 *Results*

5.3.1 Progesterone concentrations

Using logistic regression procedures no significant effects of herd, season,

Table 5.2. Odds ratios of variables included in the final logistic regression model for high plasma progesterone (P4) concentrations (≥9 ng/ml) on Day 42 of gestation.

Factor	Class	High plasma P4		Odds ratio	95% CI[a]	*P*-value
		n	%			
Multiple corpora lutea	One	35/143	24.5	*Referent*		
	Two or more	26/56	46.4	3.01	1.52 – 5.99	0.002
Interaction season x milk production	Warm x Low production	28/64	43.8	2.86	1.45 – 5.63	0.002
	Warm x High production	14/72	19.4	*Referent*		
	Cool x Low production	10/32	31.3	1.61	0.54 – 4.74	0.39
	Cool x High production	9/31	28.1	1.22	0.43 – 3.46	0.71
Interaction Parity x PAG-category	Primiparous x Low PAG	4/26	15.4	0.35	0.11 – 1.16	0.085
	Primiparous x Medium PAG	5/22	22.7	*Referent*		
	Primiparous x High PAG	12/23	52.2	2.73	1.08 – 6.89	0.034
	Multiparous x Low PAG	16/58	27.6	1.56	0.86 – 11.92	0.32
	Multiparous x Medium PAG	12/41	29.3	1.67	0.76 – 11.6	0.15
	Multiparous x High PAG	12/29	41.4	1.99	0.89 – 6.78	0.09

Likelihood ratio test, 262.55; 4 d.f., P<0.0001. Hosmer and Lemeshow goodness-of-fit test, 2.292; 5 d.f., P=0.809 (the model fits).

[a]Confidence interval for the odds ratio.

PAG-1 concentrations, parity and days in milk on plasma progesterone concentrations were found. Table 5.2 shows the odds ratios of the variables and interactions included in the final model. Based on the odds ratio, cows with multiple corpora lutea were three times more likely to have high plasma progesterone concentrations than cows with a single corpus luteum. Interactions between season and milk production and between parity and PAG-1 levels were also found to be significant. Low producing cows during the warm season were 2.86 times more likely to have high progesterone than the remaining cows. Primiparous cows showing high PAG-1 (>4 ng/ml) were 2.73 times more likely to have high progesterone than the remaining cows.

5.3.2 Fetal loss

Of the 199 pregnancies, 25 (12.6%) suffered early fetal loss: 22/136 (16.2%) during the warm season and 3/63 (4.8%) during the cool season, all in cows without additional corpora lutea. Herd, parity, days in milk, twin pregnancy, season, milk production, additional corpora lutea, plasma progesterone and PAG-1 concentrations did not affect fetal loss. Only the interaction season x additional corpora lutea showed an effect on early fetal loss (Table 5.3). Based upon the odds ratio, cows without an additional corpus luteum were 3.67 times more likely to suffer fetal loss during the warm season than during the cool season.

5.4 Discussion

To our knowledge, this report is the first description of the factors that affect plasma concentrations of progesterone at the start of the early fetal period in high producing dairy cows. Our main findings were that low milk production during the warm season and the presence of two or more corpora lutea were related to increased plasma progesterone. A positive association was furthermore found between high concentrations of progesterone and high concentrations of PAG-1 in primiparous cows. Finally, an additional corpus luteum was confirmed as a preventive factor for early fetal loss.

A close correlation between dry matter intake and milk production has been observed in lactating dairy cows (Harrison *et al*., 1990). Liver blood flow and steroid metabolism have been described as acutely elevated by feed consumption

Table 5.3. Odds ratios and pregnancy loss rates of variables included in the final logistic regression model for early fetal loss.

Factor	Class	Pregnancy loss n	Pregnancy loss %	Odds ratio	95% CI[a]	*P*-value
Interaction season x additional corpus luteum	Warm x no aCL	22/122	18.0	3.67	1.57-12.83	0.04
	Cool x no aCL	3/53	5.7	*Referent*		

[a]Confidence interval for the odds ratio.

both in lactating and non-lactating cows (Sangsritavong *et al.*, 2002). Wiltbank *et al.* (2006) proposed that the changes in reproductive physiology noted in high-producing cows may be due to high blood flow to the liver resulting from high dry matter intake, which causes low circulating concentrations of steroid hormones. Our results partly support this hypothesis in pregnant cows. During the warm season, high plasma progesterone was related significantly only to low milk production. During the cool period, both high and low producers, just as high producers during the warm period, were less likely to have high progesterone. In warm weather, dry matter intake is reduced (De Rensis and Scaramuzzi, 2003; West, 2003). This probably means that steroid metabolism was reduced in low producers, because dry matter intake was minimized during the warm season, whereas despite heat stress, a higher feed intake was necessary for high producers to maintain milk production during this period. We suggest that plasma progesterone concentrations at the onset of the fetal period in high milk producing dairy cows can be correlated negatively with milk production.

The corpus luteum is the major source of progesterone in the cow during the two first terms of gestation (Niswender *et al.*, 2000; Sawyer, 1995). Pregnant cows with two or more corpora lutea were three times more likely to have high plasma progesterone concentrations than their partners with a single corpus luteum. This highlights both the positive effects of an additional corpus luteum on maintaining gestation (Garcia-Ispierto *et al.*, 2006; López-Gatius *et al.*, 2002; López-Gatius *et al.*, 2004b) and the idea that progesterone concentrations are suboptimal during the early fetal period in high producing dairy cows (López-Gatius *et al.*, 2004a). Our results contrast with those described in previous studies in which similar plasma progesterone concentrations were recorded for animals with a single corpus luteum and with two corpora lutea, both in non-lactating non-pregnant cows (Mann *et al.*, 2007) and in pregnant cows in gestation Week five (Starbuck *et al.*, 2004). Many are the factors involved in the formation and regulation of luteal structures (Niswender *et al.*, 2000; Schams and Berisha, 2004). Here, we identified several factors that affect plasma progesterone during a stage of gestation in which progesterone is mainly produced by luteal function. Hence, factors related to luteal function could explain the discrepancies among studies. This issue requires further investigation.

In cows, trophoblastic binucleate cells from the early conceptus synthesize substantial amounts of glycoproteins related to pregnancy (PAG). Some of these (PAG), specifically the PAG-1 subgroup are released into the maternal circulation at the time of implantation (i.e., Day 25) rising steadily as gestation proceeds and peaking just before parturition (Green *et al.*, 2005; Mialon *et al.*, 1993; Zoli *et al.*, 1992). Although their function remains unknown, PAG-1 concentrations in maternal blood have been used for both pregnancy diagnosis and as a marker of fetal/placental well-being (Skinner *et al.*, 1996; Szenci *et al.*, 1998a,b). The relationship between plasma progesterone concentration and trophoblast secretory properties (PAG-1 secretion) was addressed in a recent study (Ayad *et al.*, 2007), in which a link was detected between high progesterone concentrations and high plasma PAG-1 concentrations. Similar results were found in the present study, but only in primiparous cows. Thus, primiparous cows showing high PAG-1 (>4 ng/ml) were 2.73 times more likely to have high progesterone than the remaining cows. Multiparous cows produce more milk than primiparous cows. Therefore, as noted above, a higher dry matter intake related to high milk production could promote a decrease in plasma concentrations of both progesterone and PAG-1. In effect, plasma PAG-1 concentrations in high producing dairy cows correlates negatively with milk production (López-Gatius *et al*., 2007a).

Our analysis of the factors affecting early fetal loss, revealed that only the interaction season x additional corpora lutea was significant. An additional corpus luteum was a powerful preventive factor for early fetal loss during the warm season, with no pregnancy losses recorded in gestations with additional corpora lutea (neither during the cool season), in agreement with previous studies (Garcia-Ispierto *et al*., 2006; López-Gatius *et al*., 2002; López-Gatius *et al*., 2004b). Despite this, other factors such as twinning or warm season, previously related to a high risk of fetal loss, showed no effect here. Probably, the small size of the study population and the high number of independent bulls used masked the possible effects of other negative factors. The sire of the fetus is often a major factor related to pregnancy loss (López-Gatius *et al*., 2002; López-Gatius *et al*., 2004b; Pegorer *et al*., 2007; Starbuck *et al*., 2004).

Given the key role of progesterone in maintaining gestation, it is important that we identify possible factors affecting concentrations of progesterone in pregnant cows, especially in high-producing dairy cows, in which pregnancy losses are increasing dramatically. We suggest that high producing cows may be more likely to have suboptimal concentrations of progesterone, therefore affecting the success of gestation. The presence of two or more corpora lutea was correlated with increased concentrations of progesterone. Thus, strategies that induce the formation of an additional corpus luteum may help increase progesterone concentrations in high producing dairy cows. For example, treatment with GnRH at AI and 12 days later increased the likelihood of a cow producing an additional corpus luteum by a factor of 3.7 (López-Gatius *et al*., 2006).

As an overall conclusion, our results indicate that milk production, the presence of two or more corpora lutea and plasma PAG-1 concentrations can affect plasma progesterone concentrations at the onset of the fetal period. Under our working conditions, an additional corpus luteum had a pronounced effect in reducing early fetal loss during the warm period.

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6

Pregnancy Patterns during the Early Foetal Period in High Producing Dairy Cows Treated with GnRH or Progesterone

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6 PREGNANCY PATTERNS DURING THE EARLY FOETAL PERIOD IN HIGH PRODUCING DAIRY COWS TREATED WITH GNRH OR PROGESTERONE

Abstract

In order to explore pregnancy patterns in high producing dairy cows treated with GnRH or progesterone at pregnancy diagnosis (Days 28-34), two consecutive experiments were designed. In Experiment 1, cows bearing a single embryo were randomly assigned to a PRID (n = 40; cows fitted with a progesterone releasing intra-vaginal device for 28 days), GnRH (n = 40; cows receiving GnRH) or Control (n = 26; untreated cows) group. PRID treatment led to a rise in plasma progesterone concentrations in the 7 days following the onset of treatment compared to the other two groups. In Experiment 2, in which we also examined twin pregnancies, animals were randomly assigned to PRID (n = 312) or GnRH (n = 294) treatment groups. Treatments were the same as described for Experiment 1. Logistic regression procedures revealed that in cows with a single corpus luteum, the probability of pregnancy loss between the first (Days 28-34) and second (Days 56-62) pregnancy diagnosis decreased by a factor of 0.51 in the PRID group compared to the GnRH group. However, in cows with two or more corpora lutea, PRID treatment increased the likelihood of pregnancy loss by a factor of three, compared to GnRH treatment. In cows carrying twins, the conceptus reduction rate was higher (P = 0.02) for the GnRH (36%) than for the PRID (16.4%) group. Formation of a new corpus luteum was recorded in 17.7% of cows in the GnRH group. Our results indicate that compared to GnRH treatment, progesterone treatment given at pregnancy diagnosis in high producing dairy cows, reduced by a factor of 0.51 and increased by a factor of 3 the probability of pregnancy loss in cows with one single or with two or more corpora lutea, respectively, and reduced the conceptus reduction rate in cows carrying twins. The practical implications of our findings are that in herds with a high incidence of early fetal loss of a non-infectious nature, treatment at the time of pregnancy diagnosis with PRID in cows with one corpus luteum and with GnRH in cows with two or more corpora lutea should offer considerable benefits.

Keywords: Early fetal loss; Progesterone supplementation; GnRH; Twins; Dairy cattle

6.1 *Introduction*

During the past few decades, dairy herds have been under ever-increasing pressure to improve their productive efficiency, yet many aspects of the reproductive process cow remain inefficient (Lucy, 2001; López-Gatius, 2003; Royal *et al*., 2000). Getting the cow pregnant in a reasonable time is an important issue, but it is also essential that pregnancy progresses safely to term. The embryonic period of gestation extends from conception to the end of the differentiation stage (about 42 days), and the fetal period runs from Day 42 to parturition (Committee on Bovine Reproductive Nomenclature, 1972). Most pregnancy losses occur during the early embryonic period (Ball, 1997; Hanzen *et al*., 1999; Vanroose *et al*., 2000). However, under the intensive management systems used today, the incidence of early fetal loss is increasing (Forar *et al*., 1995), and rates may exceed 12% (Day *et al*., 1995; Markusfeld-Nir, 1997; Santos *et al*., 2004).

Early fetal loss peaks between 45 and 60 days of gestation (López-Gatius *et al*., 2004b), when the association between mother and conceptus is still not fully developed (Curran *et al*., 1986; Riding *et al*., 2008). Several cow and management factors of a non-infectious nature have been related to pregnancy loss during this period in our area (Labèrnia *et al*., 1996; López-Gatius *et al*., 2002) and elsewhere (Gabor *et al*., 2008; Grimard *et al*., 2006; Santos *et al*., 2004; Silke *et al*., 2002). The presence of an additional corpus luteum (number of corpora lutea exceeding number of embryos) has been identified as a main preventive cow factor for pregnancy loss (Bartolome *et al*., 2006; Bech-Sàbat *et al*., 2008; Garcia-Ispierto *et al*., 2006; López-Gatius *et al*., 2002; López-Gatius *et al*., 2004b). In the cow, the corpus luteum is the main source of progesterone, at least during the first 200 days of pregnancy (Niswender *et al*., 2000; Sawyer, 1995). Progesterone, the key hormone of gestation, is required for maternal support of conceptus survival and development (Spencer *et al*., 2004). Pregnancy maintenance has been positively correlated with plasma concentrations of progesterone on Week 5 of gestation (Starbuck *et al*., 2004), and progesterone concentrations have been reported to affect the secretory functions of the trophoblast and pituitary during the first trimester of gestation (Ayad *et al*., 2007). However, one of the consequences of high milk production is an increased metabolic rate linked to a greater intake of dry matter. This process reduces plasma concentrations of steroid hormones such as progesterone (Sangsritavong *et al*., 2002). In effect, milk production can negatively affect plasma progesterone concentrations at the onset of the fetal period (Bech-Sàbat *et al*., 2008). Twenty-eight days of intra-vaginal progesterone supplementation starting from Days 36 to 42 of gestation reduces the incidence of losses in high producing dairy cows (López-Gatius *et al*., 2004a). Therefore, it seems reasonable to suppose that one of the causes of early fetal loss in high producing dairy cows could be suboptimal concentrations of progesterone, either due to increased progesterone catabolism, reduced luteal function, or both. Thus, strategies that induce the formation of an additional corpus luteum may help to increase progesterone concentrations and are hypothesized to result in a more prolonged action on maintenance of gestation than that a progesterone-based treatment regimen. For example, treatment with GnRH at AI and 12 days later has been found to increase the likelihood of a cow producing an additional corpus luteum by a factor of 3.7 (López-Gatius *et al*., 2006), whereas GnRH treatment on Day 27 of gestation significantly increases the chances of an accessory corpus luteum on Day 45 of gestation (Bartolome *et al*., 2006). In both these studies the presence of an additional corpus luteum reduced the risk of fetal loss (Bartolome *et al*., 2006; López-Gatius *et al*., 2006), however treatment did not promote pregnancy maintenance. Probably, the number of cows in each treatment group (89 (Bartolome *et al*., 2006) and 152 (López-Gatius *et al*., 2006) pregnancies) was too small to establish a clear positive effect of GnRH treatment in reducing early fetal loss. Because progesterone treatment is more expensive and laborious at the farm level than GnRH we compared the two treatments to gain further insight into some of the problems related to early fetal loss and information on possible differences in response levels to each treatment. The aim of the present study was to determine plasma progesterone concentrations, early fetal loss and GnRH-induced corpus luteum formation in high producing dairy cows treated with GnRH or progesterone at the time of pregnancy diagnosis (Days 28-34). Cow and management factors previously found to be related to early fetal loss were also assessed.

6.2 Materials and methods

6.2.1 Cattle and herd management

Two consecutive experiments were performed on two commercial dairy herds in northeastern Spain, selected because of their high incidence of early fetal loss over a 13-month period (February 2007 to February 2008). The herds comprised a mean of 88 (Herd 1) and 585 (Herd 2) mature Holstein-Friesian cows, with a mean annual milk production for this period of 9260 and 11250 kg per cow, respectively. The cows, reared within the herds, calved all year round, were milked two (Herd 1) or three (Herd 2) times per day and fed complete rations. Feeds consisted of cotton-seed hulls, barley, corn, soybean and bran, and roughage, primarily corn, barley and alfalfa silages and alfalfa hay. Rations were in line with NRC recommendations (National Research Council, 2001). All the animals were tested free of tuberculosis and brucellosis and bred by artificial insemination using semen from 35 bulls of proven fertility. Mean annual culling rates for the study period were 20% for Herd 1 and 31% for the Herd 2.

The herds were maintained on a weekly reproductive health program. The reproductive tract of each animal was examined by palpation per rectum 30-36 days postpartum to check for normal uterine involution and the morphology of ovarian structures. Reproductive disorders diagnosed at this time were treated until resolved or until culling. The voluntary waiting period from calving to first AI established for these herds was 45 days post-partum. Only cows free of detectable reproductive disorders were inseminated.

6.2.2 Pregnancy diagnosis, number and viability of embryos and number of corpora lutea

Pregnancy was diagnosed by transrectal ultrasonography from Days 28 and 34 after insemination (first pregnancy diagnosis) using a portable B-mode ultrasound scanner (Scanner SonoSite 180 PLUS Vet equipped with a 10-5 MHz transducer; SonoSite, Bothell, WA, USA). Scanning was performed along the dorsal/lateral surface of each horn. The viability of embryos was determined by detecting their heartbeat. The presence of twins was established through the observation of two embryos in different positions within one uterine horn on two screen scans, two embryos simultaneously present on screen, or one embryo in each uterine horn.

Each ovary was scanned in several planes by moving the transducer along its surface to identify luteal structures, and the number of corpora lutea recorded for each cow. Pregnancies in which the number of corpora lutea exceeded the number of embryos were recorded as pregnancies with an additional corpus luteum.

Only cows carrying live embryos, either singletons or twins, were included in the study. We excluded from the study, cows with: dead embryos (two singleton and five twin pregnancies); abnormalities in the reproductive tract (i.e. uro/pneumovagina, n = 9); contralateral pregnancies (embryo located in the uterine horn contralateral to the corpus luteum, n = 1); monozygotic twin pregnancies (n = 3); and triplets (n = 1). Confirmation of pregnancy was performed on Days 56-62 post-insemination by ultrasound (second pregnancy diagnosis). At this time, conceptus reduction in twin pregnancies (presence of one single live fetus) and formation of a GnRH-induced corpus luteum for the GnRH-treated pregnancies were recorded in cows not suffering pregnancy loss. The possible formation of a new corpus luteum was also assessed in the PRID group. Fetal loss was registered when the second diagnosis proved negative.

6.2.3 Experimental design

6.2.3.1 Experiment 1

This experiment was performed in Herd 2, on animals bearing a single embryo. Cows found to be pregnant on first pregnancy diagnosis (Days 28-34 days post-insemination) were assigned in chronological order of diagnosis to a "PRID group" (n = 40) or "GnRH group" (n = 40) and treated at the moment of pregnancy diagnosis. A control group was established by including in the experiment an untreated cow for each three treated animals (n = 26). In the PRID group, cows were fitted with a progesterone releasing intra-vaginal device (PRID, containing 1.55 g of progesterone, CEVA Salud Animal, Barcelona, Spain) for 28 days. In the GnRH group, cows received a GnRH dose (100 μg i.m.; Cystoreline, CEVA Salud Animal).

Blood samples were withdrawn from the coccygeal vein of all animals immediately after the first pregnancy diagnosis into heparinized vacuum tubes (BD VacutainerTM, Becton, Dickenson and Company, Plymouth, UK). A second blood sample was obtained 7 days after the first. The samples were centrifuged (10 min at 1600 x g) within 30 min of collection, and plasma stored at -20 ºC until assayed.

Plasma progesterone concentrations were determined by a direct radioimmunoassay (RIA) method (without extraction) as previously described in detail (López-Gatius *et al.*, 2007). The minimum detection limit of the P4-RIA technique used was 0.15 ng/ml, and intra-assay coefficients of variation were 5.1% and 10.5%, respectively. Plasma progesterone determinations were performed only on samples derived from animals not suffering pregnancy loss between the first and second pregnancy diagnosis.

The following data were recorded for each animal: pregnancy loss, treatment, herd, lactation number, interval from parturition to conception, additional corpus luteum, milk production (average of the 3 days prior to pregnancy diagnosis), and plasma progesterone values.

6.2.3.2 Experiment 2

Experiment 2 was performed on both Herds 1 and 2. Animals found to be pregnant on first pregnancy diagnosis were assigned in chronological order of diagnosis to a "PRID group" (n = 312) or "GnRH group" (n = 294). Treatments were the same as described for Experiment 1.

The data recorded for each animal were the same as in Experiment 1, except plasma progesterone values, plus: service sire, twin pregnancy and date of treatment. In the geographical area of study, there are only two clearly differentiated climate periods: warm (May to September) and cool (October to April) (Labernia *et al.*, 1998). Reproductive variables are generally significantly impaired in the warm period (López-Gatius, 2003). For this reason, treatment dates were used to analyze the effect of the season (warm versus cool period) on pregnancy loss.

6.2.4 Data analysis

6.2.4.1 Experiment 1

The effects of lactation number, days in milk at conception, milk production at pregnancy diagnosis, number of corpora lutea, treatment and possible interactions of paired factors on plasma progesterone concentration were analyzed

Table 6.1. Productive status of cows for Experiments 1 and 2.

	PRID Group [a]	GnRH Group [b]	Control [c]	Total
	Mean ± SD (ranges)	Mean ± SD (ranges)	Mean ± SD (ranges)	Mean ± SD (ranges)
Experiment 1				
Number of animals	32	35	19	86
Days in milk at conception	163 ± 89 (47 – 356)	152 ± 101 (51 – 431)	137 ± 32 (90 – 212)	153 ± 85 (47 – 431)
Lactation number	2.3 ± 1.4 (1 – 6)	2.4 ± 1.5 (1 – 6)	2.7 ± 1.1 (1 – 4)	2.5 ± 1.4 (1 – 6)
Milk production at PD (kg)	41 ± 6 (31 – 60)	39 ± 9 (21 – 60)	44 ± 7 (32 – 53)	41 ± 8 (21 – 60)
Experiment 2				
Number of animals	312	294	-	606
Herd 1	38	37	-	75
Herd 2	274	257	-	531
Days in milk at conception	141 ± 88 (45 – 568)	138 ± 88 (45 – 657)	-	140 ± 88 (45 – 657)
Lactation number	2.4 ± 1.4 (1 – 7)	2.5 ± 1.5 (1 – 6)	-	2.4 ± 1.4 (1 – 7)
Milk production at PD (kg)	38 ± 8 (15 – 61)	38 ± 8 (17 – 60)	-	38 ± 8 (15 – 61)

[a] Cows fitted at pregnancy diagnosis (days 28 – 34) with a Progesterone Releasing Intravaginal Device (PRID, containing 1.55 g of progesterone) for 28 days.
[b] Cows treated with 100 μg GnRH i.m. at pregnancy diagnosis (days 28 – 34).
[c] Cows with no treatment.

by GLM repeated measures analysis of variance using the SPSS package, version 13.0 (SPSS Inc., Chicago, IL, USA). Lactation number, days in milk and milk production were introduced as covariables in the analysis.

6.2.5.2 Experiment 2

The relative contribution of each factor to the probability of pregnancy loss was determined by logistic regression procedures. Since the presence of two corpora lutea had a positive clear positive effect on plasma progesterone concentrations in Experiment 1, we performed logistic regression analysis for animals with one single or two or more corpora lutea. Pregnancy loss was considered as the dependent variable for each group. Season (warm period), high milk production (≥40 kg at pregnancy diagnosis) and treatment (dichotomous variables, where "1" denotes presence and "0" denotes absence); lactation number and days open (continuous variables); and herd and service sire (class variables) were considered as factors in the analysis. Twin pregnancy was included as a dichotomous variable in the group of animals with two or more corpora lutea.

Regression analyses were performed using the SPSS package version 13.0 (SPSS Inc., Chicago, IL, USA) according to the method of Hosmer and Lemeshow (1989). Basically, this method consists of five steps as follows: (1) preliminary screening of all variables for univariate associations; (2) construction of a full model, using all the significant variables resulting from the univariate analysis; (3) stepwise removal of nonsignificant variables from the full model and comparison of the reduced model with the previous model for model fit and confounding; (4) evaluation of interactions among variables; (5) and assessment of model fit using Hosmer-Lemeshow statistics. Variables with univariate associations showing P-values < 0.25 were included in the initial model. Modeling was continued until all the main effects or interaction terms were significant according to the Wald statistic at P < 0.05.

The probability of differences between treatments for the conceptus reduction rate in twin pregnancies between the first and the second pregnancy diagnosis was determined by the X^2-test, with 0.05 as the level of significance.

6.3 Results

Table 6.1 shows the variables related to the productive status of the cows for the PRID, GnRH and Control groups for Experiments 1 and 2: days in milk at conception, lactation number and milk production at pregnancy diagnosis.

6.3.1 Experiment 1

In groups PRID, GnRH and Control, 32, 35 and 19 cows, respectively, did not suffer pregnancy loss and were included in the analysis. Of these animals, 7

Table 6.2. Plasma progesterone concentrations (ng/mL) determined 7 days apart for each treatment in Experiment 1.

	PRID Group (n = 32)	GnRH Group (n = 35)	Control (n = 19)	Total (n = 86)
	Mean[c] ± SD (ranges)	Mean ± SD (ranges)	Mean ± SD (ranges)	Mean ± SD (ranges)
[P4] – 1[a]	5.3[A] ± 2.3 (1.8 – 11.8)	4.8 ± 1.7 (2.6 – 9.3)	5.2 ± 1.9 (2.6 – 9.1)	5.1 ± 2.0 (1.8 – 11.8)
[P4] – 2[b]	6.8[B] ± 3.0 (2.1 – 16.4)	5.0 ± 1.7 (2.1 – 8.9)	5.2 ± 2.0 (2.5 – 11.1)	5.7 ± 2.5 (2.1 – 16.4)

[a]Plasma progesterone concentrations at first pregnancy diagnosis (days 28 – 34)
[b]Plasma progesterone concentrations seven days later.
[c]Different superscripts within the column denote significant differences detected by the GLM repeated measures analysis of variance (P<0.001).

(21.9 %), 12 (34.3 %) and 8 (42.1 %) cows had two corpora lutea, respectively. Table 6.2 shows progesterone concentrations for each group on pregnancy diagnosis and 7 days later. Analysis by GLM repeated measures revealed no effect of lactation number, days in milk at conception and milk production on progesterone concentrations at the two time points. Table 6.3 provides the variables included in the final model for factors affecting progesterone concentrations. Significant effects on both sampling days and an interaction between day of sampling and treatment were observed. This interaction produced a significantly different pattern of progesterone concentrations between the two sampling days in animals receiving different treatments. When these effects where studied, PRID treatment showed a positive effect on the rise in progesterone concentrations (within-subjects) and on these concentrations (between-subjects) compared to the other two groups, in which no significant differences where detected (Fig. 6.1A). Significant positive effects were also detected in cows with two corpora lutea (Table 6.3 and Fig. 6.1B).

Table 6.3. Main model of the GLM repeated measurement analysis for factors affecting progesterone concentrations in Experiment 1.

Subject effects	Factor	d.f.	F	P-value
Within	Sampling days	1	8.6	0.004
	Days x treatment	2	6.4	0.003
	Days x number of CL	1	0.76	0.387
Between	Treatment	2	4.0	0.022
	Number of CL	1	4.7	0.034

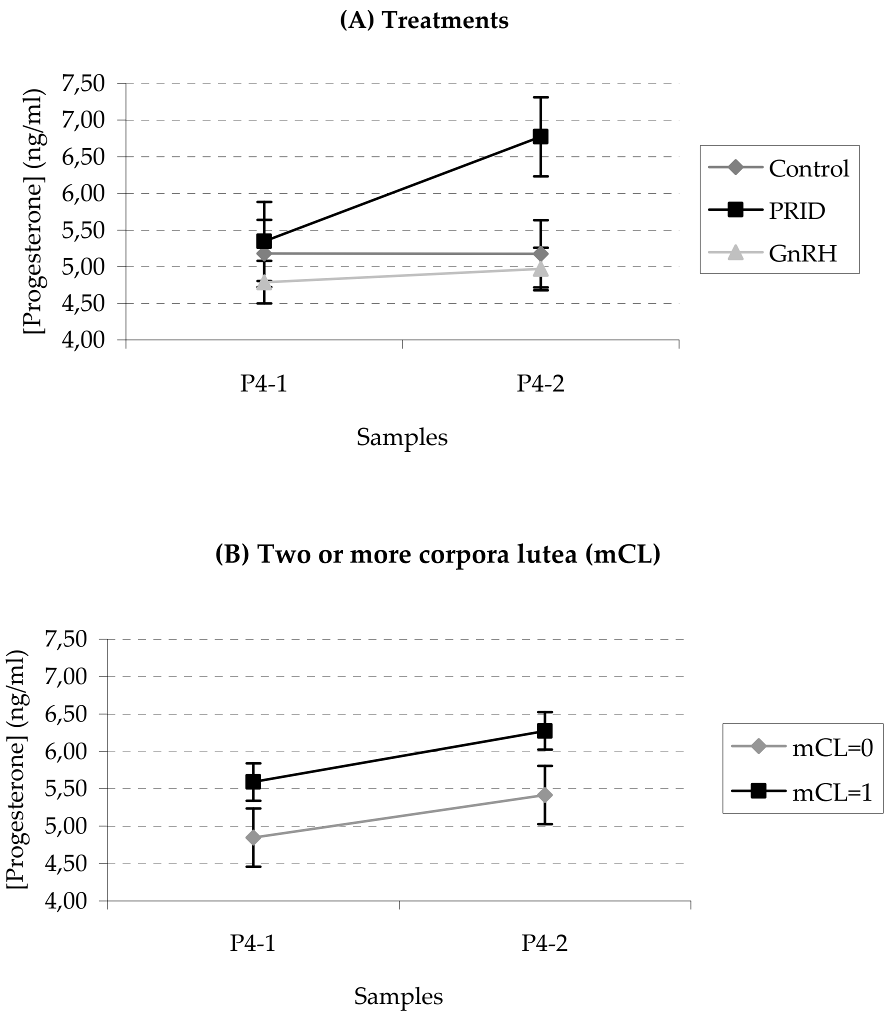

Figure 1. Progesterone concentrations recorded at pregnancy diagnosis (P4-1) and seven days later (P4-2) stratified according to the variables included in the final model of GLM repeated measures: Treatment (A) and presence of two or more multiple corpora lutea (B) (mean values ± S.D.).

6.3.2 Experiment 2

Table 6.4 summarizes pregnancy types (number of embryos and corpora lutea) detected on first pregnancy diagnosis for each treatment, and rates of conceptus reduction and neo-formation of corpora lutea detected on second pregnancy diagnosis.

Table 6.4. Number of pregnancies in Experiment 2 classified by number of embryos and number of corpora lutea detected on the first pregnancy diagnosis (FPD, Days 28-34); and rates of pregnancy loss, CL neo-formation and conceptus reduction registered at the second pregnancy diagnosis (SPD, Days 56-62).

	FPD	SPD			
	n	Pregnancy loss (%)[a]	Pregnancy maintenance	CL neo-formation (%)[b]	Conceptus reduction (%)[a,c]
PRID[d]					
Singletons	226	17 (7.5)	209	-	-
Singletons with aCL	31	3 (9.7)	28	-	-
Twins	55	11 (20)	44	-	9 (16.4)[f]
Twins with aCL	-	-	-	-	-
Total	312	31 (9.9)	281	-	
GnRH[e]					
Singletons	212	29 (13.7)	183	44 (24)	-
Singletons with aCL	32	1 (3.1)	31	1 (3.2)	-
Twins	49	4 (8.2)	45	1 (22.2)	18 (36.7)[g]
Twins with aCL	1	0	1	-	1
Total	294	34 (11.6)	260	46 (17.7)	

CL = Corpus Luteum; aCL = additional Corpus Luteum (pregnancies with CL number exceeding the number of embryos).
[a]With respect to the total number of pregnancies in the same group.
[b]With respect to the total animals with positive SPD in the same group.
[c]Significant differences detected by the chi-square test ([f,g] $P = 0.02$).
[d]Cows fitted with a Progesterone Releasing Intravaginal Device (PRID, containing 1.55 g of progesterone) at FPD for 28 days.
[e]Cows receiving a GnRH dose (100 μg i.m.; Cystoreline) at FPD.

6.3.2.1 Pregnancy loss

Overall pregnancy loss detected between the first and second pregnancy diagnoses were 10.7 % (65/606): 9.9 % (31/312) and 11.5 % (34/294) for the PRID and GnRH groups, respectively. Using logistic regression procedures, no significant effects of herd, lactation number, number of services, service sire, days in milk at conception, milk production at treatment on fetal loss were found for animals with a single or with two or more corpora lutea. Season had not effect in cows with two or more corpora lutea. Table 6.5 shows the pregnancy loss rates and odds ratios of the variables finally included in the logistic model for cows with a single or two or more corpora lutea. No interactions were found. In cows with a single corpus luteum (all animals carrying singletons), based on the odds ratio, the probability of pregnancy loss was 0.51.fold lower in cows receiving PRID treatment than cows treated with GnRH; whereas pregnancy loss was 2.2 times more likely for cows treated during the warm than during the cool season. Fetal loss recorded in cows fitted with PRID were 4.3% (6/140) and 12.8% (11/86) for the cool and warm

Table 6.5. Odds ratios and pregnancy loss rates of variables included in the final logistic regression model for early fetal loss.

Factor	Class	Pregnancy loss		Odds ratio	95 % CI[a]	P-value
		n	%			
Pregnancies with one single corpus luteum (n = 438)[b]						
Treatment	PRID	17/226	7.5	0.51	0.27 – 0.95	0.035
	GnRH	29/212	13.7	Reference		
Season of treatment	Warm	25/165	15.2	2.17	1.17 – 4.04	0.014
	Cool	21/273	7.7	Reference		
Pregnancies with two or more corpora lutea (n = 168)[c]						
Treatment	PRID	14/86	16.3	2.99	1.03 – 8.73	0.045
	GnRH	5/82	6.1	Reference		

[a]Confidence interval for the odds ratio
[b]Likelihood ratio test, 10.487; 2 d.f., P = 0,005. Hosmer and Lemeshow goodness-of-fit test, 0.995; 2 d.f., P = 0,608.
[c]Likelihood ratio test, 4.511; 1 d.f., P = 0,034.

periods, respectively; whereas early fetal loss rates in cows receiving GnRH treatment were 11.3% (15/133) and 17.7% (14/79) for the cool and warm periods, respectively.

In the group of cows with two or more corpora lutea (63 cows carrying singletons with an additional corpus luteum plus 105 cows carrying twins with two or more corpora lutea), based on the odds ratio, cows treated with PRID were three times more likely to suffer fetal loss than cows treated with GnRH. Early fetal losses recorded in cows carrying singletons with an additional corpus luteum were 9.7% (3/31) and 3.1% (1/32) for the PRID and GnRH groups, respectively; whereas these figures were 20% (11/55) and 8% (4/50), respectively, for cows with twin pregnancies.

Since the presence of twin pregnancies could mask results of animals with two or more corpora lutea, a further analysis was performed on all animals with a single versus two or more corpora lutea, including an additional corpus luteum and twin pregnancy (dichotomous variables) as possible factors affecting pregnancy loss. In this analysis, the model was not adjusted.

6.3.2.2 Conceptus reduction

Of the 105 twin pregnancies registered, 15 (14.3%) ended in pregnancy loss and 27 (25.7%) suffered conceptus reduction. Table 6.4 shows conceptus reduction rates for each treatment. The conceptus reduction rate was significantly higher (P = 0.02) for the GnRH (18/50, 36%) than for the PRID (9/55, 16.4%) group.

6.3.2.3 GnRH-induced corpora lutea

In pregnant cows on second pregnancy diagnosis, formation of a new corpus luteum was not detected in any cow in the PRID group, whereas this event was observed in 17.7% (46/260) of cows in the GnRH group: 45 in singleton and 1 in twin pregnancies (Table 6.4).

6.4 Discussion

Our study was designed to compare pregnancy patterns in cows treated with progesterone (PRID) or GnRH at the time of pregnancy diagnosis (28-34 days of gestation) in herds with a high incidence of early fetal loss. Its main findings were: (1) PRID treatment led to a significant increase in plasma progesterone concentrations 7 days after starting treatment, whereas similar progesterone levels were recorded after 7 days of treatment in the GnRH treated- and untreated-Control cows; (2) in cows carrying singletons with a single corpus luteum, progesterone supplementation reduced the likelihood of early fetal loss by a factor of 0.51 compared to cows receiving GnRH; (3) in cows with two or more corpora lutea, progesterone supplementation increased the likelihood of pregnancy loss three times compared to cows receiving GnRH; and (4) the conceptus reduction rate was significantly higher in the GnRH group than in PRID groups of cows carrying twins.

Intra-vaginal progesterone supplementation during the late embryonic period gave rise to an increase in plasma progesterone concentrations during the first week of treatment. Commercially available devices for progesterone supplementation for cattle have been tested in ovariectomized (Vancleeff *et al*., 1992), anestrous post-partum (McDougall *et al*., 2004; Nation *et al*., 2000) and cyclic cows (Keefe *et al*., 2006), and during the early embryonic period (Larson *et al*., 2007; Lynch *et al*., 1999; Mann *et al*., 2006) and lead to increased milk and plasma progesterone concentrations. Although we could not assess endogenous luteal progesterone production, our results indicate that the net balance of plasma circulating progesterone during the late embryonic period was clearly augmented in animals receiving progesterone supplementation. In contrast, GnRH treatment was unable to increase plasma progesterone concentrations during the first week of treatment. The pharmacological basis for the therapeutic use of GnRH or its synthetic analogs derives mainly from its physiological effect of stimulating the release of LH from the anterior pituitary gland, enhancing the action of an existing corpus luteum or inducing the formation of a new corpus luteum (Peters, 2005). Our results suggest that to increase progesterone availability, the effect, if any, of GnRH on the existing corpus luteum was weak, at least in the short term. Although the GnRH group in Experiment 2 showed a 17.7% rate of GnRH-induced corpora lutea, these new corpora lutea would not have been functional when early fetal loss occurred. This was probably why the risk of losses was two times higher (1/0.51) for the GnRH-treated cows with a single corpus luteum compared to their counterparts receiving PRID. Similar results have been described by Bartolome *et al*. (2006): despite a 43.6% incidence of GnRH-induced corpora lutea following GnRH treatment on Day 27 of gestation, GnRH treatment did not reduce the rate of fetal loss compared to untreated cows. In the latter study (Bartolome *et al*., 2006), increases in plasma progesterone levels were observed on Day 45 of gestation in GnRH-treated cows; a GnRH effect that probably occurred too late to maintain pregnancy.

Our results support the idea that most pregnancy losses during the early fetal period are linked to suboptimal plasma progesterone concentrations in high producing dairy cows. This could indeed be the case in pregnancies with a single corpus luteum (72.3%: 438/606). However, cows with two or more corpora lutea unexpectedly showed an opposite response to cows with a single corpus luteum. Progesterone supplementation increased the risk of early fetal loss three times in these cows, compared to GnRH treatment. This suggests that plasma concentrations of progesterone need to be within a precise range during the late embryo- early fetal period and both below optimal and above optimal plasma progesterone concentrations seem to be lead to pregnancy failure. Under our working conditions, the presence of two corpora lutea was related to increased progesterone levels, in agreement with a previous study (Bech-Sàbat *et al*., 2008). Thus, progesterone supplementation seems not necessary in cows with two or more corpora lutea. The question is why should progesterone supplementation be a problem in cows with two corpora lutea? Is it because of feedback mechanisms of progesterone? Or could there be excessive immune-depression related to the too high plasma progesterone levels?

As discussed earlier, intra-vaginal progesterone supplementation has proved valuable to increase plasma progesterone concentrations (Keefe *et al*., 2006; Larson *et al*., 2007; Lynch *et al*., 1999; Mann *et al*., 2006; McDougall *et al*., 2004; Nation *et al*., 2000; Vancleeff *et al*., 1992). However, when a PRID was inserted in cows with two corpora lutea, it seems that high plasma progesterone levels mediated an excessive negative LH feedback to the hypothalamus, reducing endogenous luteal progesterone production. Progesterone supplementation could be related to suppressed endogenous production of progesterone when administered during Days 10-17 after insemination (Robinson *et al*., 1989). Conversely, progesterone, among its other functions, is one of the main regulators of the immune-modulation needed for the successful establishment of the conceptus (a semi-allograft) in the uterus (Druckmann and Druckmann, 2005; Szekeres-Bartho *et al*., 2001), gestation being the outcome of a delicate balance among numerous molecules (Spencer and Bazer, 2004; Wolf *et al*., 2003). Probably, progesterone supplementation impairs embryo-maternal communication in cows with two corpora lutea. Further, pregnancy losses for twin pregnancies were 20 % and 8 % for the PRID and GnRH groups, respectively. Since according to previous studies, the twin pregnancy loss rate in our area ranges from 21 to 29% (Garcia-

Ispierto *et al*., 2006; López-Gatius *et al*., 2002; López-Gatius *et al*., 2004b; López-Gatius and Hunter, 2005), we cannot rule out positive effects of GnRH on twin pregnancies. In effect, GnRH treatment clearly promoted pregnancy maintenance in cows suffering conceptus reduction, which was observed in 37% and 14% of the GnRH- and PRID-treated cows, respectively.

Reduction of the embryo or fetal number in twin pregnancies involves modification and gradual dissolution of a single conceptus without compromising the viability of its co-twin (López-Gatius and Hunter, 2005). The way in which GnRH treatment induced pregnancy maintenance in cows suffering conceptus reduction is another question that arises. The recruitment of large numbers of white blood cells, local release of cytokines and release of prostaglandins from uterine tissues should not be discarded. The effectiveness of GnRH was probably due to an indirect antiluteolytic effect. The release of LH following GnRH treatment can cause either luteinization or atresia of antral follicles in the ovary and disruption of the estradiol control of the uterus that leads to PGF2α release (Thatcher *et al*., 1989).

Although the effects of cow and management factors on pregnancy loss have been the focus of previous studies (Labèrnia *et al*., 1996; López-Gatius *et al*., 2002; López-Gatius *et al*., 2004a,b), given their large variation ranges, the possible effects of these factors were analyzed in the present study. Our results were consistent with those reported in previous studies: no significant effects of herd, lactation number, number of services, days in milk at conception and milk production at treatment on fetal loss for animals with a single or two or more corpora lutea. In cows with one corpus luteum, pregnancy loss was 2.2 times more likely in cows treated during the warm season than cows treated during the cool season. This result reinforces previous findings in our geographical area in which the likelihood of pregnancy loss was from 1.6 to 4 times higher in cows that became pregnant in the warm than in the cool season (Garcia-Ispierto *et al*., 2006; López-Gatius *et al*., 2004a-c).

As an overall conclusion, our findings indicate that treatment with progesterone at the time of pregnancy diagnosis in high producing dairy cows, significantly increases plasma progesterone concentrations 7 days after starting treatment, compared to cows receiving GnRH. In turn, the likelihood of early fetal loss is reduced by a factor of 0.51 in cows carrying singletons with one corpus luteum whereas in cows with two or more corpora lutea, progesterone supplementation increases the likelihood of pregnancy loss three times and reduced the conceptus reduction rate in cows carrying twins. The practical implications of our findings are that in herds with a high incidence of early fetal loss of a non-infectious nature, treatment at the time of pregnancy diagnosis with PRID in cows with one corpus luteum and with GnRH in cows with two or more corpora lutea should offer considerable benefits.

6.5 *References*

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7 Effects of a Progesterone-Based Oestrus Synchronization Protocol in 51- to 57-Day Postpartum High Producing Dairy Cows

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7 Effects of a Progesterone-based Oestrus Synchronization Protocol in 51- to 57-Day Postpartum High Producing Dairy Cows

Abstract

The aim of this study was to investigate the effect of applying a progesterone-based oestrus synchronization protocol at 51-57 days postpartum in high-producing dairy cows. The data analysed were derived from 1345 lactating cows. Cows between 51 and 57 days postpartum were assigned to the groups: Control, PRID (receiving a progesterone-releasing intravaginal device for 9 days, and prostaglandin F2α 24 h before PRID removal) or GnRH-PRID (the same as the PRID group plus GnRH at PRID insertion). Oestrus was detected by using pedometers and confirmed by examination of the genital tract at AI. Oestrous and conception rates before days 71-77 postpartum, pregnancy loss in early pregnant cows or the cumulative conception rate registered on day 120 postpartum were considered as the dependent variables in four consecutive logistic regression analyses. Based on the odds ratios, the oestrous rate increased by a factor of 1.73 in cows showing oestrus before treatment for each unit increase in the number of previous oestruses; decreased by a factor of 0.44 in the control group with respect to the treatment groups; and by a factor of 0.61 in cows without luteal structures at treatment with respect to cows with corpora lutea. The conception rates of cows inseminated before days 71-77 postpartum remained similar across the groups, whereas the likelihood of pregnancy loss for cows becoming pregnant during this period was 0.11 times lower in the PRID group than in the control. Based on the odds ratio, the likelihood of a higher cumulative conception rate on day 120 postpartum: increased in cows showing oestrus before treatment by a factor of 1.41 for each unit increase in the number of previous oestruses, was reduced 0.56-fold in control cows compared with treated cows, and was also reduced by a factor of 0.98 for each kilogram of milk production increase recorded at treatment. In conclusion, although oestrous synchronization programmes performed in this study did not improve fertility, cows treated with progesterone could be inseminated earlier than untreated cows, such that the treatments increased the cumulative pregnancy rates determined on day 120 postpartum. In addition, fewer pregnancy losses were observed in early pregnant cows in the PRID group than the GnRH-PRID group.

Keywords: progesterone-based oestrus synchronization, pregnancy, pregnancy loss, dairy cows

7.1 Introduction

High-producing dairy cows are usually subfertile under current production systems. The reasons for the lower fertility of modern dairy herds are multifactorial and not entirely linked to increased milk production (García-Ispierto *et al*., 2007; Lucy, 2001; López-Gatius, 2003; López-Gatius *et al*., 2006). The slow recovery of reproductive competence during the postpartum period is a major limitation to the success of subsequent reproductive management programmes, with anoestrus as a main postpartum disorder (Yániz *et al*., 2008). In effect, over the past few years the postpartum anovulatory period at the start of the voluntary waiting period has been getting longer (López-Gatius, 2003; Opsomer *et al*., 2000; Yániz *et al*., 2008). Progesterone-based treatments involving the use of a progesterone-releasing intravaginal device (PRID) have significantly improved the synchronization of oestrus and the probability of pregnancy after first insemination in anovulatory cows (Larson and Ball, 1992; Lòpez-Gatius *et al*., 2001; López-Gatius *et al*., 2004c; López-Gatius *et al*., 2008; Nebel and Jobst, 1998). Indeed, low fertility in cattle has been related to low progesterone concentrations in the oestrus cycle preceding insemination (Folman *et al*., 1990), and it seems that exposure to exogenous progesterone during the cycle prior to insemination is beneficial for fertility (Wehrman *et al*., 1993).

As high-producing dairy cows show a higher metabolism rate than lower producers, including clearance of steroid hormones such as 17β-oestradiol and progesterone (Sangsritavong *et al*., 2002), we hypothesized that plasma progesterone concentrations around the time of the milk production peak might be critical for the success or failure of reproduction in high-producing dairy cows. The aim of this study was to investigate the effects of two progesterone-based oestrus synchronization treatments given on 51-57 days postpartum in high-producing dairy cows.

7.2 Materials and Methods

7.2.1 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 1345 mature cows over the period December 2005 to October 2008. Briefly, herd management included the following common features: the use of pedometers, housing in free stalls with concrete slatted floors and cubicles, fans and water sprinklers for the warm season, rigorous postpartum checks, the same reproductive health programme, confirmation of oestrus at AI by palpation per rectum and the fact that most AI (over 90%) were performed by veterinarians.

The mean annual culling rate was 28%. Mean annual milk production for the herds over the study period was 10.650 kg/cow. 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 (National Research Council, 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 established for postpartum nutrition and controls, and 7–20 days 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 days.

7.2.2 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 hypocalcemia and ketosis (the latter, diagnosed during the first or second week postpartum), retained placenta (foetal 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). The herds were maintained on a weekly reproductive health programme. This involved examining the reproductive tract of each animal by ultrasound from 30 to 36 days postpartum to check for normal uterine involution and ovarian structures. Reproductive disorders diagnosed at this time such as pyometra or ovarian cysts were treated until resolved. Detectable intrauterine cloudy fluid was interpreted as pyometra. An ovarian cyst was diagnosed when a follicular structure larger than 20 mm in diameter was detected in either or both ovaries in the absence of a corpus luteum and uterine tone. Cows with a retained placenta or primary metritis were always treated with oxytetracycline boluses introduced into the uterus. Prostaglandin F2α was applied at the end of treatment for a retained placenta and primary metritis.

7.2.3 Treatments

Cows from 51 to 57 days postpartum with no uterine nor ovarian disorders detected by ultrasound were randomly assigned to the Control (untreated n = 473), PRID (treated with PRID n = 455) or GnRH-PRID (treated with PRID plus GnRH n = 417) groups. 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 days and these animals were also given prostaglandin F2α (25 mg dinoprost i.m.; Enzaprost, CEVA Santé Animale, Libourne, France) 24 h before PRID removal (López-Gatius *et al.*, 2005; López-Gatius *et al.*, 2006; López-Gatius *et al.*, 2008). Cows in the PRID-GnRH group received the same treatment as the PRID group plus GnRH (100 μg i.m.; Cystoreline®, CEVA Santé Animale, Libourne, France) at PRID insertion (Lòpez-Gatius *et al*., 2001; López-Gatius *et al*., 2004c). Only healthy cows (with no signs of mastitis, lameness or digestive disorders) were included in the study. Ovarian structures were registered at treatment. Corpora lutea were defined as small or normal in size if they were smaller, or equal to or larger than 15 mm (mean of the maximum and minimum diameters), respectively.

7.2.4 Insemination and pregnancy diagnosis

Oestrus was detected using a pedometer system (AfiFarm System; SAE Afikim, Kibbutz Afikim, Israel). Walking activity values were recorded at the milking parlour (three times daily) and analysed automatically using the herd management computer programme. A walking activity more than 80% above the mean activity recorded in the previous 2 days was taken as the lower limit for a cow to be considered in oestrus. As these herds have been observed to show a very significant relationship between increased activity and fertility provided this increase is 80% to 993% (López-Gatius *et al*., 2005), values lower than 80% were not considered oestrous signs. We also took into account previous individual information concerning oestrous detection. For example, if a cow showed a 120% increase in activity yet during its two last oestrous 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 examination programme. The cows were finally inseminated after oestrus had been confirmed by examination of the genital tract and vaginal fluid (Lopez-Gatius and Camon-Urgel, 1991). 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 days post-insemination. Pregnancy was confirmed by palpation per rectum 90–96 d post-insemination. Foetal loss was recorded when the 90–96 days-diagnosis proved negative. Cows diagnosed as not pregnant and cows with no oestrous signs before days 71-77 in milk were included in a weekly reproductive program and were inseminated either following a specific treatment (López-Gatius *et al*., 2008) or during natural oestrus. Data from cows suffering any clinical disease before day 120 in milk (open cows) or before Day 90 of gestation (pregnant cows) were withdrawn from the study. All gynaecological examinations and pregnancy diagnoses were performed by the same veterinarian.

7.2.5 Data collection and analysis

In our geographical region, there are only two clearly differentiated weather periods: warm (May–September) and cool (October–April) (Labernia *et al*., 1998; López-Gatius, 2003). Thus, treatment dates were used to analyse the effect of treatment season on the oestrous response, fertility and pregnancy losses.

The following data were recorded for each animal upon treatment: herd, previous number of oestruses, age (primiparous vs multiparous), ovarian structures (follicle, small or normal corpus luteum), treatment (0 = Control, 1= PRID, 2= GnRH-PRID), season of treatment (cool versus warm), oestrous rate within 20 d of treatment onset (before days 71-77 in milk), milk production at treatment (mean production of 3 days before treatment), AI date, semen providing bull, AI technician, pregnancy diagnosis 28–34 d following AI either subsequent to treatment or following inseminations performed before day 120 in milk, and foetal loss following a positive pregnancy diagnosis. The factors parity and season of treatment were coded as dichotomous variables, where 1 denotes presence and 0 denotes absence. Herd, treatment, ovarian structures, semen providing bull and AI technician, were considered as class variables. Milk production and number of oestruses before treatment were considered continuous variables.

Four binary logistic regression analyses were performed. The dependent variables considered in these four analyses, respectively, were early oestrous rate (before days 71-77 in milk), early conception rate for cows inseminated before days 71-77 in milk, pregnancy loss in early pregnant cows, or cumulative conception rate on day 120 in milk.

Regression analyses were conducted according to the method of 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 $P < 0.25$ were included in the initial model. We continued modelling 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, and probabilities between 0.05 and 0.10 were discussed as tendencies.

7.3 *Results*

Over the study period, means for the number of inseminations and the intervals parturition-first insemination and parturition-gestation were 1.4 ± 0.7 AI, 73.7 ± 14.1 days, and 83.4 ±17.7 days, respectively (mean ± SD). Oestrous response rates before 71-77 days in milk and conception rates for cows inseminated during

Table 7.1. Odds ratios of the variables included in the final logistic regression model for factors affecting the estrous rate following treatment

Factor	Class	Oestrous rate		Odds ratio	95% CI[a]	P-value
		n	%			
Herd	1	89/106	84.0	6.24	3.3-10.6	<0.001
	2	629/951	66.1	1.85	1.3-2.6	<0.001
	3	141/288	49.0	Reference		
Treatment	PRID	325/455	71.4	2.6	1.9-3.4	<0.001
	GnRH-PRID	294/417	70.5	2.5	1.8-3.3	<0.001
	Control	240/473	50,7	Reference		
Ovarian structures	No CL	391/684	57.2	0.6	0.5-0.8	0.001
	Small CL	260/365	71.2	1.5	0.8-1.8	0.39
	Normal-size CL	208/296	70.3	Reference		
Previous number of oestruses	Continuous	859/1345	63.9	1.73	1.4-2.2	<0.001

CL: corpus luteum
Likelihood ratio test = 243.60; 70df, P=0.0001. Hosmer and Lemeshow Goodness-of-fit test = 21.6; 3 df, P = 0.87.
[a]Confidence interval for the odds ratio.

this period were 63.9% and 31.9%, respectively. Mean milk production at treatment was 44.9 ± 9.8 kg (25-72 kg).

7.3.1 Early oestrous rates

Logistic regression analysis indicated no significant effects of season of treatment and milk production on the oestrous response rate. No significant interactions were found. Based on the odds ratios, the likelihood of an oestrous response following treatment was: higher in herds 1 and 2 by factors of 6.25 and 1.85, respectively, compared with herd 3; higher by a factor of 1.73 in cows expressing prior oestrus, for each unit increase in the number of oestruses, compared with cows with no previous oestruses; and higher by factors of 2.6 and 2.5 in the PRID and PRID-GnRH group, respectively, using the control group as reference. The likelihood of an oestrous response was 0.61 times lower in cows without luteal structures at the start of treatment, than in animals with a normal sized CL used as reference (Table 7.1).

7.3.2 Conception rates

No significant effects were found of herd, number of previous oestruses, parity, ovarian structures, milk production, treatment, season of treatment, semen providing bull and AI technician on the likelihood of pregnancy in cows inseminated before days 71-77 postpartum. No significant interactions were

Table 7.2. Odds ratios of the variables included in the final logistic regression model for factors affecting pregnancy loss in early pregnant cows (first AI before 71-77 d postpartum).

Factor	Class	Pregnancy losses		Odds ratio	95% CI[a]	P-value
		N	%			
Treatment	PRID	6/107	5.6	0.11	0.03 – 0.4	<0.001
	GnRH-PRID	13/96	13.5	0.47	0.21 – 1.01	0.055
	Control	19/73	25.6	Reference		

Likelihood ratio test = 243.60; 70df, P=0.0001. Hosmer and Lemeshow Goodness-of-fit test = 21.6; 3 d.f., P =1.
[a]Confidence interval for the odds ratio.

observed. Conception rates were 30% (72/240), 33% (107/325) and 33% (97/294) in the control, PRID and GnRH-PRID groups, respectively.

7.3.3 Pregnancy losses in early pregnant cows

No significant effects were found of herd, number of previous oestruses, parity, ovarian structures, milk production, season of treatment and semen providing bull on pregnancy loss in early pregnant cows. Based on the odds ratio, the likelihood of pregnancy loss following first insemination was 0.11 times lower in cows treated with a PRID, compared with control cows (Table 7.2).

7.3.4 Cumulative conception rates

No significant effects were detected of ovarian structures, season of treatment, semen-providing bull, number of previous oestruses and parity on the cumulative conception rate. Based on the odds ratio, the likelihood of pregnancy by day 120 was higher in herd 2 (1.85-fold), compared to the remaining herds, higher in cows in the PRID (2.5-fold) and GnRH-PRID (2.4-fold) groups compared with control cows, and lower by a factor of 0.98 for each kilogram increase in milk production. No significant interactions were found (Table 7.3).

7.4 Discussion

A progesterone-based treatment, either adding GnRH or not to the protocol, given from 51 to 57 days postpartum proved useful in our high-producing dairy cows. The conception rates of cows inseminated before days 71-77 postpartum remained similar across the groups. Although, untreated cows took 9 days longer than the progesterone-treated cows to express oestrus, treatment increased early oestrous rate and cumulative conception rate. In addition, treatment increased the oestrous rate and cumulative conception rate recorded on day 120, and decreased pregnancy losses in cows becoming pregnant before day 71-77

Table 7.3. Odds ratios of the variables included in the final logistic regression model for factors affecting cumulative conception rate registered on Day 120 postpartum.

Factor	Class	Conception rate		Odds ratio	95% CI	P-value
		n	%			
Herd	1	53/106	50.0	1.58	0.99 – 2.5	0.06
	2	515/951	54.2	1.85	1.4 – 2.4	<0.001
	3	110/288	38.3	Reference		
Treatment	PRID	258/455	56.7	2.5	1.8 – 3.1	0.001
	GnRH-PRID	227/417	54.4	2.4	1.7 – 3.1	0.001
	Control	193/473	40.8	Reference		
Milk production	Continuous	678/1345	50.4	0.98	0.96 – 0.98	<0.001

CL: corpus luteum
Likelihood ratio test = 243.60; 70df, P=0.0001. Hosmer and Lemeshow Goodness-of-fit test = 21.6; 3 df, P = 0.76.
[a]Confidence interval for the odds ratio.

postpartum. The best results were obtained following the progesterone-based protocol without GnRH.

Cows that have not regained oestrous cyclicity by 60-day postpartum or before the first postpartum AI show a reduced probability of pregnancy per AI and increased pregnancy losses following first insemination (Cerri *et al*., 2004; Chebel *et al*., 2006; Santos *et al*., 2004; Stevenson *et al*., 2006). Because in high-producing dairy cows first postpartum ovulation is often delayed, methods designed to induce cyclicity in anoestrous cows should improve reproductive performance (Yániz *et al*., 2004). In our study, cows expressing a prior oestrus before 51-57 days postpartum showed a 1.73-times greater likelihood of oestrus. In addition, it has been reported that cows with delayed ovarian cyclicity beyond 56 days postpartum have a lower conception rate (Yamada *et al*., 2003; Yániz *et al*., 2006). Thus, cows that quickly resume oestrus following parturition may be described as cyclic and are more sensitive to treatment, probably owing to the presence of a corpus luteum in the ovary.

The presence of ovarian structures at the beginning of treatment affected the oestrous response rate. Specifically, cows with a corpus luteum on day 0 were 1.6 times (1/0.6) more likely to show oestrus than cows lacking luteal structures. This means that in most cows with a CL, ovarian function was normal. Some authors have reported highest conception rates in response to a synchronization protocol starting 5 to 11 days after oestrus (Vasconcelos *et al*., 1999). Thus, short exposure to endogenous or exogenous elevated progesterone concentrations during the postpartum period seems to be important for the expression of oestrus and for subsequent normal luteal function (Rhodes *et al*., 2003). Cows with normal ovarian cyclicity at the beginning of treatment may show a better response to treatment, improving the oestrous rate.

All the cows included in our study produced more than 25 Kg of milk at treatment. This means that the animals may have had a severe to moderate negative energy balance 51-57 days postpartum, which has been related to ovarian dysfunction, cystic ovarian follicle (Jolly *et al.*, 1995) and reduced oestrous behaviour (Lopez *et al.*, 2004). As a consequence of increased blood flow and hepatic metabolism, high producing cows typically show elevated clearance of steroid hormones from the circulation, leading to lower progesterone and oestradiol levels compared to nulliparous heifers (Sartori *et al.*, 2002; Wolfenson *et al.*, 2004). Usually, treatment is applied just at or immediately after the time of peak of milk production, which is when the cow is likely to need more progesterone. In this study, progesterone treatment was found to increase the oestrous rate by a factor of 2.6 (PRID group) or 2.5 (GnRH-PRID group). The mechanism of action of exogenous progesterone in re-establishing cyclicity is not fully understood, but progesterone is thought to recover the number of oestrogen receptors in the medial basal hypothalamus, re-establishing oestradiol responsiveness and provoking a pre-ovulatory LH surge (Gumen and Wiltbank, 2005). In effect, exogenous progesterone during peak milk production seems to be a useful tool for avoiding a reduced oestrous rate. This was reflected by our finding of improved fertility, in that cumulative fertility recorded on day 120 was 2.5 and 2.4 times higher in the progesterone-treated groups (PRID and GnRH-PRID, respectively) than the untreated cows.

No significant effect of treatment 2 was detected on pregnancy loss. Conversely, treatment 1 was linked to pregnancy losses reduced by a factor of 0.11.

In a previous study, we detected the effect of progesterone supplementation in reducing early foetal loss at the end of the late embryo period (López-Gatius *et al.*, 2004a). However, it remains to be determined if progesterone treatment before gestation helps to maintain gestation during the early foetal period. The main effect of exogenous progesterone even when given on day 51-57 postpartum could be to supplement the role of a possible sub-functional corpus luteum by raising the concentration of progesterone in the systemic circulation. Circulating concentrations of progesterone during the cycle immediately prior to insemination have been related to embryo survival (Diskin *et al.*, 2006). The authors of this last study noted positive correlation between progesterone concentration on the day of PGF2α-induced luteolysis and the subsequent embryo survival rate (Diskin *et al.*, 2006). Low progesterone concentrations in the cycle preceding oestrus are most likely to affect the subsequent embryo survival rate by provoking early maturation of the oocyte, thus compromising its ability to continue normal embryo-early foetal development after fertilization. In contrast, high progesterone levels have been reported to diminish myometrial activity (Csapo and Pulkkinen, 1978), possibly promoting the development of the maternal side of the placenta, especially the function of the caruncules. Our findings prompt the question of why PRID plus GnRH treatment failed to reduce pregnancy losses as did PRID alone. Although fewer losses were recorded in the GnRH-PRID group than control group, just a tendency could be registered, the difference was not significant (P = 0.055). This is a conflicting result so that a greater study population should confirm significant differences or not. Anyway, although plasma progesterone concentration were not determinated herein, a possible explanation could stem from the fact that GnRH plus PRID treatment could increase plasma progesterone levels, compared to PRID treatment alone. High-dose progesterone has been proposed to impair LH support of the dominant follicle and induce atresia and follicle turnover (Adams *et al*., 1992). GnRH treatment induces a surge of LH and FSH (Chenault *et al*., 1990) favouring ovulation or luteinization of large ovarian follicles present at the time of treatment (Macmillan and Thatcher, 1991). The apparent antagonism anti- (progesterone) pro-LH (GnRH) may furthermore create a conflict in the follicular wave and embryo development could be affected as a consequence.

Pregnancy loss in the control group was 25.6%, which is high compared with previous rates recorded in our geographical area (Garcia-Ispierto *et al*., 2006; López-Gatius *et al*., 2002; López-Gatius *et al*., 2004a,b). This high rate could be related to the metabolic stress of cows becoming pregnant during the peak time of milk production emphasizing the potential beneficial effects of progesterone treatment in cows suffering different types of stresses.

In conclusion, although our progesterone-based synchronization protocols did not improve fertility, treated cows could be inseminated earlier than untreated cows such that by day 120 postpartum cumulative conception rates were higher. Moreover, pregnancy losses in early pregnant cows were reduced in response to PRID treatment.

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8 Therapeutic Approaches to Pregnancy Loss of Non-Infectious Cause during the Late Embryonic/Early Foetal Period in Dairy Cattle. A Review.

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8 THERAPEUTIC APPROACHES TO PREGNANCY LOSS OF NON-INFECTIOUS CAUSE DURING THE LATE EMBRYONIC/EARLY FOETAL PERIOD IN DAIRY CATTLE. A REVIEW.

Abstract

The problem of pregnancy loss during the late embryonic/early foetal period due to non-infectious causes in dairy cattle is on the rise, especially in high milk producing cows. This review discusses recent therapeutic approaches designed to reduce the incidence of early foetal loss. These strategies are based on the fact that low plasma concentrations of progesterone are commonly related to foetal loss. Progesterone supplementation during the late embryonic/early foetal period has proven useful in curtailing losses, mainly in single pregnancies, whereas a more positive effect of treatment with GnRH than progesterone has been found in twin pregnancies. Therapeutics is not necessary in cows with an additional corpus luteum. In practical terms, suggestions include recording the number of embryos and corpora lutea on first pregnancy diagnosis, and checking for normal pregnancy progression on Day 60 post-insemination.

Keywords: Early foetal loss, progesterone, gonadotrophins, dairy cattle

8.1 *Introduction*

Establishing and maintaining pregnancy in mammals is dependent upon a complex process of conceptus-mother endocrine-immune crosstalk involving the participation of many molecules (Gootwine, 2004; Wolf *et al*., 2003). This process is especially important during early pregnancy. The role of progesterone, the so-called "hormone of pregnancy" is unequivocal in this molecular network (Inskeep, 2004; Spencer *et al*., 2004). Embryo attachment and implantation, placentation, and the close relationship established between the conceptus and maternal reproductive tissues are all critical steps that are highly sensitive to several forms of stress. Pregnancy loss of a non-infectious nature during the late embryonic/early foetal period is increasing in dairy cattle, especially in high production systems in which the incidence can exceed 20% (Bartolome *et al*., 2005b-d; Cartmill *et al*., 2001a,b; Grimard *et al*., 2006). This review discusses recent therapeutic approaches designed to reduce the incidence of early foetal loss based on the fact that low plasma concentrations of progesterone have been linked to foetal loss.

8.2 *Pregnancy loss during the late embryonic/early foetal period*

In cattle, the embryonic period of gestation extends from fertilization to the end of the differentiation stage (approximately 42 days), and the foetal period spans from Day 42 of gestation to parturition (Committee on Bovine Reproductive Nomenclature, 1972). Placentation is completed approximately on day 60 of gestation (Curran *et al*., 1986; Riding *et al*., 2008), when pregnancy is considered to be firmly established and the chances of failure are reduced (Ball, 1997). Although most pregnancy losses in dairy cattle occur during the early embryonic period (Hanzen *et al*., 1999; Inskeep and Dailey, 2005; Peters, 1996; Santos *et al*., 2004a), the risk of late embryo/early foetal loss increases under conditions of intensive management (Forar *et al*., 1995; Lopez-Gatius *et al*., 2009; Santos *et al*., 2004a). Earlier studies reported a level of loss after implantation of approximately 5-8% (Ball, 1997), whereas more recent reviews show pregnancy losses before day 90 of pregnancy of 10-12% (Lopez-Gatius *et al*., 2009; Santos *et al*., 2004a). On the other hand, rates of losses are highly variable, and some reports, especially on highly intensive managed herds or under stress situations, describe pregnancy loss rates > 20% (Bartolome *et al*., 2005b-d; Cartmill *et al*., 2001a; Grimard *et al*., 2006) and even 40% (Cartmill *et al*., 2001b).

Early foetal loss is caused by multiple factors (Vanroose *et al*., 2000). Management- and cow related factors of a non-infectious nature such as season, parity, semen-providing bull and twin pregnancies have all been described to affect early foetal loss in high producing dairy cows in northeastern Spain (Labèrnia *et al*., 1996; López-Gatius *et al*., 1996; López-Gatius *et al*., 2002; López-Gatius *et al*., 2004b,c) and elsewhere (Chebel *et al*., 2004; Gabor *et al*., 2008; Grimard *et al*., 2006; Jousan *et al*., 2005; McDougall *et al*., 2005; Pegorer *et al*., 2007; Rhinehart *et al*., 2009; Santos *et al*., 2009; Santos *et al*., 2004a; Silke *et al*., 2002; Silva-Del-Rio *et al*., 2009; Starbuck *et al*., 2004). Diet can also affect embryo survival and foetal development (McEvoy *et al*., 2001; Santos *et al*., 2004a; Shore *et al*., 1998). In warm countries such as Spain, summer heat stress dramatically increases foetal losses (Garcia-Ispierto *et al*., 2006; López-Gatius *et al*., 2004a-c; Santolaria *et al*., 2010). In fact, early foetal loss, occurring between days 40 and 50 of gestation (López-Gatius *et al*., 2004b; Santos *et al*., 2004a), is today one of the most common complications of gestation in high producing dairy cows, in which more than 90% of pregnancy losses following pregnancy diagnosis can occur before 90 days of gestation (López-Gatius *et al*., 2002; López-Gatius *et al*., 2004b).

8.3 *Role of progesterone in maintaining pregnancy during the first trimester*

Progesterone is crucial to establish and support a pregnancy. The biological activities of progesterone involve several modes of action (Arck *et al*., 2007; Falkenstein *et al*., 2000; Mahesh *et al*., 1996), which include genomic pathways (regulating gene expression mainly via nuclear receptors) and non-genomic pathways (affecting signal transduction cell responses mainly via plasma membrane receptors) (Bishop and Stormshak, 2008; Edwards, 2005; Gellersen *et al*., 2009; Schmidt *et al*., 2000; Stormshak and Bishop, 2008). Although the cell and molecular mechanisms regulating early conceptus development and implantation are still not fully understood, it is known that progesterone and progesterone-dependent factors are essential for orchestrating the histotrophic environment necessary for conceptus growth and development (Bazer, 1975; Garrett *et al*., 1988; Geisert *et al*., 1988; Morris and Diskin, 2008). Implantation and placentation are also dependent on progesterone secretion (Spencer and Bazer, 2002; Spencer *et al*., 2007). Besides its endocrine effects, and especially during these latter processes during which there is increasing contact between the conceptus and maternal immune cells, progesterone acts as an "immunosteroid". A requirement for successful pregnancy is that the uterine mucosa tolerates the allogenic embryo without compromising its immune mechanisms against microbial agents. In this regard, progesterone plays a major role in the immune-modulation of gestation, blocking T-cell lymphopoiesis during pregnancy and controlling the bias towards a pregnancy protective milieu (Arck *et al*., 2007; Druckmann and Druckmann, 2005; Hansen, 1998; Szekeres-Bartho *et al*., 2001).

Plasma concentrations of progesterone affect conceptus survival and growth during early pregnancy (Mann and Lamming, 1999). Thus, progesterone levels recorded in week five of pregnancy have been correlated with subsequent gestation maintenance (Starbuck *et al*., 2004) and have also been observed to affect the secretory functions of the trophoblast and pituitary during the first trimester of pregnancy (Ayad *et al*., 2007).

8.4 Progesterone, milk production and early foetal loss

Wiltbank *et al*. (2006) hypothesised that the changes in reproductive physiology noted in high producing cows could be due to a high rate of steroid metabolism. Briefly, this rationale arises from the fact that the higher dry matter intake of high producing dairy cows results in higher blood flow to the digestive tract, which in turn increases blood flow to the liver and enhances steroid metabolism (Rabiee *et al*., 2001, 2002; Sangsritavong *et al*., 2002; Vasconcelos *et al*., 2003). Accordingly, studies have shown that milk production is negatively related to plasma progesterone concentrations at the onset of the foetal period (Bech-Sàbat *et al*., 2008; Rhinehart *et al*., 2009). Hence, it seems reasonable to suppose that one of the causes of early foetal loss in high producing dairy cows could be sub-optimal concentrations of progesterone, either due to increased progesterone catabolism and sub-luteal function, or to both these factors.

8.5 *The presence of an additional corpus luteum prevents pregnancy loss*

The corpus luteum is the major source of progesterone in the cow at least during the first 200 days of gestation (Niswender *et al.*, 2000; Sawyer, 1995). In pregnancies in which more corpora lutea than the number of embryos are detected, the presence of these additional corpora lutea has been strongly linked to reducing foetal loss. For example, according to data from five studies (Bech-Sàbat *et al.*, 2008; Garcia-Ispierto *et al.*, 2006; López-Gatius *et al.*, 2002; López-Gatius *et al.*, 2004b; López-Gatius *et al.*, 2006), 1.7% of 363 pregnant cows with an additional corpus luteum suffered foetal loss, compared to a 9.9% rate of pregnancy loss recorded in 3643 pregnant animals with no additional corpus luteum. The odds ratio for pregnancy loss was 0.12-0.32 for cows with an additional corpus luteum with respect to those without one. In three of these five studies, all cows carrying singletons with two corpora lutea did not undergo any pregnancy loss (Bech-Sàbat *et al.*, 2008; López-Gatius *et al.*, 2002; López-Gatius *et al.*, 2006). Further, although plasma progesterone concentrations are highly variable among cows, compared to cows with a single corpus luteum, cows with two or more corpora lutea were three times more likely to have high plasma progesterone concentrations (≥ 9 ng/ml) on day 42 of gestation (Bech-Sàbat *et al.*, 2008), and these type of animals also showed higher plasma progesterone concentrations between day 28 and 34 of gestation (Bech-Sàbat *et al.*, 2009).

8.6 *Treatment with gonadotrophins (GnRH or hCG)*

As the presence of an additional corpus luteum is a strong preventive factor for foetal loss, strategies inducing the formation of an additional corpus luteum should help to increase plasma progesterone levels in high producers. Gonadotrophins such as hCG and GnRH have the physiological effect of inducing the sudden release of LH from the anterior pituitary gland (Chenault *et al.*, 1990; Rajamahendran and Sianangama, 1992). However, although treatment with GnRH at AI (López-Gatius *et al.*, 2006) or during the early embryonic period (López-Gatius *et al.*, 2006; Sterry *et al.*, 2006; Stevenson *et al.*, 2006; Stevenson *et al.*, 2007), and with GnRH or hCG at pregnancy diagnosis (Bartolome *et al.*, 2006; Stevenson *et al.*, 2008) clearly increases the number of additional corpora lutea, it has not been found to reduce foetal loss in any of the studies performed. Similarly, using GnRH on Day 21 or 23 of gestation as a resynchronization strategy, Chebel *et al.* (2003) and Bartolomé *et al.* (2005d), found no effects on subsequent pregnancy losses in cows that were pregnant at the time of treatment. In beef cows without a primary corpus luteum, the ability of corpora lutea to maintain pregnancy was greater when induced after rather than before day 36 of gestation (Bridges *et al.*, 2000). In a further study (Fischer-Tenhagen *et al.*, 2008), hCG treatment on day 4 post-AI was linked to pregnancy maintenance on days 27-30 of gestation, but only during the warm season. Finally, Bartolomé *et al.* (2005a) detected no effect of GnRH treatment on day 5 or 15 post-AI on the pregnancy rate recorded on day 55, but treatment applied both days increased the risk of foetal losses. Thus, not only a lack of effect but possible negative effects on pregnancy maintenance should be contemplated following gonadotrophin treatments. The reasons why GnRH- or hCG-induced corpora lutea are unable to mitigate losses remain to be clarified.

To reduce follicular growth, as another suspected cause of increased embryo loss (Inskeep, 2004), Santos *et al*. (2004b) replaced the second GnRH i.m. dose in an Ovsynch protocol for a subcutaneous deslorelin implant. A tendency of reduced early foetal loss was noted loss using a 450 μg dose but not a 750 μg dose. This strategy has also been used to overcome follicular development during heat stress, but no effects on subsequent pregnancy maintenance were detected (Silvestre *et al*., 2009).

8.7 Progesterone treatment

The use of progesterone following AI has been often proposed as a strategy to improve fertility, but has not always yielded clear results. In a meta-analysis, Mann and Lamming (1999) were able to demonstrate a significant positive effect of progesterone treatment in low fertility herds during the first week post-AI, but not during the second and third weeks.

To test the hypothesis that sub-optimal progesterone concentrations may compromise conceptus development during the early foetal period, we supplemented pregnant cows with exogenous progesterone (López-Gatius *et al*., 2004a). Treatment was started on pregnancy diagnosis, between days 36 and 42 post-insemination, with a progesterone releasing intravaginal device containing 1.55 g of progesterone, for 28 days. Based on the odds ratio, the risk of pregnancy loss was 2.4 times higher in non-treated cows than in treated ones: 12% (66/549) versus 5.3% (29/549) of losses, respectively. Similar results were obtained when the device was fitted on day 28 of gestation: 16% (16/97) losses for non-treated and 6% (6/102) losses for treated cows (Bech-Sabat *et al*., 2007). In a more recent study (Alnimer and Lubbadeh, 2008) in which progesterone was given from day 28 to 35 post-AI, treatment tended to reduce losses: 21% (36/172) losses for non-treated and 15% (19/126) losses for treated cows. These results support the hypothesis that sub-optimal progesterone concentrations in high producing dairy cows may compromise conceptus development. Under these conditions, the available data suggest that intra-vaginal progesterone supplementation can reduce the incidence of pregnancy loss during the early foetal period.

Some studies have evaluated the effect of progesterone used in oestrous synchronisation protocols before timed AI on subsequent early foetal loss. El-Zarkouny *et al*. (2004) observed reduced pregnancy losses from day 29 to 57 when progesterone treatment [controlled internal drug-releasing intravaginal insert (CIDR)] was added to the Ovsynch protocol. Yet, in other studies conducted in similar conditions this correlation could not be found (Cerri *et al*., 2009; Galvao *et* *al*., 2004; Rivera *et al*., 2005; Stevenson *et al*., 2006). Resynchronization protocols may include progesterone treatments before the expected time of re-insemination when the pregnancy status is unknown. In studies assessing early foetal loss in pregnant cows after progesterone supplementation over days 13-14 to 20-21, positive effects of treatment were noted (Chebel *et al*., 2006; El-Zarkouny and Stevenson, 2004). Furthermore, the effect of progesterone supplementation during early embryonic period on subsequent early foetal loss has also been evaluated, observing no effect (Sterry *et al*., 2006) or detecting a tendency to enhance late embryo survival (Villarroel *et al*., 2004). Yet, most studies on progesterone supplementation during synchronization or resynchronization protocols, or during the early embryonic period, were designed to assess conception rates and not early foetal loss. Thus, the number of pregnant cows in which subsequent loss could be determined in these studies has always been small. Although the hypothesis that improve early gestation could reduce the incidence of losses is interesting, more research is needed in this area.

8.8 Comparing GnRH and progesterone treatments at pregnancy diagnosis

In a recent study performed in two herds with a high incidence of foetal loss (Bech-Sàbat *et al*., 2009), intra-vaginal progesterone supplementation at pregnancy diagnosis (days 28-34) led to increased plasma progesterone concentrations during the first week of treatment, whereas GnRH treatment did not. In a second experiment in the same study (Bech-Sàbat *et al*., 2009), cows were randomly assigned to progesterone (n = 312) or GnRH (n = 294) treatment groups at pregnancy diagnosis. In cows with a single corpus luteum, the probability of pregnancy loss decreased by a factor of 0.51 in the progesterone group compared to the GnRH group. Yet, in cows with two or more corpora lutea, progesterone treatment increased the likelihood of pregnancy loss by a factor of three compared to GnRH treatment. In cows carrying twins, the conceptus reduction rate was higher for the GnRH (36%) than for the progesterone (16%) group. The effectiveness of GnRH was probably due to an indirect antiluteolytic effect. Thus, LH release following GnRH treatment can cause disruption of the oestradiol control of the uterus that leads to PGF2α release (Thatcher *et al*., 1989). A high incidence of pregnancy loss (63%) has been observed following spontaneous twin reduction in untreated cows (López-Gatius and Hunter, 2005).

8.9 The problem of twin pregnancies

Twin pregnancy is probably the main factor related to early foetal loss in high producing dairy herds. Using cows carrying singletons as reference and compiling the data from five studies (Garcia-Ispierto *et al*., 2006; López-Gatius *et al*., 2002; López-Gatius *et al*., 2004b; López-Gatius and Hunter, 2005; López-Gatius *et al*., 2006), the minimum likelihood of pregnancy loss for twin pregnancies was 3.1. As noted above, in the warm period up to 54% of losses were recorded in twin pregnancies (López-Gatius *et al.*, 2004b). In an epidemiological study on 52362 lactations, the increase in the twinning rate was linked to increased milk production (Kinsel *et al.*, 1998). In high producers, the rate of double ovulation may be over 20% (Fricke and Wiltbank, 1999) or even 25% for cows in their third or more lactation period (López-Gatius *et al.*, 2005). Since over the last few decades, genetics appears to be a major regulatory factor for twinning rates (Johanson *et al.*, 2001), and it is reasonable to suggest that increased twinning is a consequence of selection for milk yield. Apart from genetic progress, improvements in nutrition and management practices have also led to a continuous increase in milk yield. Probably, improved management at the farm level has diminished the risk of embryo loss in twin pregnancies and thus raised the twinning rate. It is therefore foreseeable that over the years to come, the twinning rate will continue to increase along with milk production.

Despite the success of GnRH treatment for twin pregnancies noted above (Bech-Sàbat *et al.*, 2009), we are presently seeing a high rate (close to 50%) of losses for ipsilateral twin pregnancies during the warm season (F. López-Gatius, unpublished data). Twin pregnancies are undesirable in dairy cattle since besides increasing the risk of early pregnancy loss, they also have negative effects such as increased abortion, dystocia, retained placenta, calf mortality, occurrence of freemartins, longer postpartum therapy, and longer calving to conception intervals (Nielen *et al.*, 1989). Such negative effects of twinning might be diminished by reducing the embryo number in dairy cows. The procedure of rupturing the amnion plus progesterone supplementation has been found to provide an open way for twin reduction in dairy cattle: four of 11 animals undergoing this procedure maintained their pregnancies (López-Gatius, 2005).

8.10 Further implications

The use of methods to decrease excessive hepatic clearance of steroid hormones without compromising dry matter intake in high producing dairy herds is a potential strategy we should not rule out. For example, it has been shown that elevated hepatic portal vein propionate increases plasma insulin in ruminants, which in turn decrease progesterone clearance (Smith *et al.*, 2006). Similarly, either providing a gluconeogenic feed-stuff (propylene-glycol) or treatment with insulin, enzymes responsible for hepatic progesterone catabolism are decreased (Lemley *et al.*, 2008). Therefore, dietary inputs and their impact on the endocrine regulation of metabolism may influence progesterone catabolism.

Some would argue that two corpora lutea are optimal in cows carrying singletons, although twins are not desirable in a herd. As mentioned previously, gonadotrophin induction of corpora lutea following AI does not seem to favour pregnancy maintenance. Since spontaneous twin reduction has been also described (López-Gatius and Hunter, 2005), assessing the factors affecting both the presence of additional corpora lutea and natural twin reduction should improve our understanding of the mechanisms involved in the maintenance of pregnancy.

Finally, and no less important, pregnant cows can show oestrus signs. For example, in the Netherlands, 4% of all calves born are attributed to an insemination before the last one (Dijkhuizen and van Eerdenburg, 1997). Accordingly, confirming true oestrus at AI in previously diagnosed pregnant cows could serve to reduce the incidence of pregnancy loss (Lopez-Gatius and Camon-Urgel, 1991; López-Gatius, 2000). Evaluation of normal pregnancy progression on day 60 after insemination is also suggested.

8.11 Concluding remarks

Large trials or meta-analytic procedures are needed to clarify the possible benefits of gonadotrophins or progesterone in the period before AI or during early pregnancy in terms of reducing subsequent early foetal loss. The benefits of GnRH administration in cows carrying twins also need to be assessed in more extensive studies. At pregnancy diagnosis (i.e. days 28-34), it is as important to record the number of corpora lutea as the number of embryos. In herds with a high incidence of early foetal loss of a non-infectious cause, treatment with progesterone in cows with one corpus luteum should offer considerable benefits. Treatment is not necessary in cows with an additional corpus luteum.

8.12 *References*

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9

General Discussion

9 GENERAL DISCUSSION

The main scope of this thesis was the analysis of factors affecting late embryonic/early foetal loss in high producing dairy cows, in order to propose and evaluate feasible, simple and practical therapeutic approaches to mitigate its increasing incidence. Pregnancy failure is a type of multifactorial disorder that is probably one of the single most economically important problems faced by the modern dairy industry. Losses during late embryonic and early foetal period, after pregnancy has been diagnosed, have been previously characterized and the following features have been established for high producing well-managed dairy herds:

- multifactorial and non-infectious causality
- the fact that they represent more than 90 % of losses after pregnancy diagnosis, becoming the most common complication of gestation
- a peak of incidence during days 45-50 of gestation
- the “natural” rate should be 5 %, although today averages are 10 % and, under particular conditions, rates may exceed 20 %

9.1 The multifactorial nature of early foetal loss

In order to summarise and analyse the effects of factors of non-infectious nature affecting late embryonic and early foetal loss in high producing dairy herds, the first study (Chapter 4) was designed, and it presented data published during the period of 1996 to 2008 analysing that reproductive disorder. Data derived from the same reproductive control program conducted by the University of Lleida on high producing dairy herds under same management conditions. This fact permitted pooling data and presenting every individual factor as proportions and ranges of odds ratios affecting foetal loss in a total of 15525 pregnancies. Results indicated that factors strongly affecting late embryonic and early foetal loss are parity (lactating cows vs heifers), semen-providing bull, warm season and twin pregnancies, whereas the presence of an additional corpus luteum was the only factor favouring the maintenance of gestation (Chapter 4). Logistic regression analyses of the following studies included in this thesis confirmed the warm season as a strong risk factor for early foetal loss (Chapters 5, 6) and the presence of an additional corpus luteum as a preventive factor (Chapter 5).

Results derived from 15525 pregnancies provide strong evidence on the relationship between factors and early foetal loss. However, we have to emphasize that these results depend on the surveillance system used to monitor these herds. Thus, many other factors not routinely measured in this surveillance system could be affecting as well. One example could be body condition score (BCS), a parameter not routinely measured but included in one of the studies analysed in Chapter 4

(López-Gatius *et al.*, 2002). In effect, cows showing a 1-unit drop in BCS from parturition to 30 days postpartum had an increased risk of early foetal loss compared to cows that maintained their BCS. Probably, an abrupt loss of the postpartum nutritional status impairs uterine involution and causes pregnancy failure when the placentomes develop. Similarly, Silke *et al*. (2002) found that 1-unit drop between days 28 and 56 of pregnancy increased 3 times the risk of pregnancy loss during this period. Controlling nutritional status, a fundamental feature for efficient production (Robinson *et al*., 2006; Roche, 2006), models for early foetal loss could be improved. BCS can provide useful information, but also other paramenters, such as non-esterified fatty acids (NEFAs), related with fat mobilization and energy balance and demonstrated to affect oocyte and early embryo (Vanholder *et al*., 2006; Leroy *et al*., 2008a,b), could probably give us more information on factors affecting early foetal loss. Furthermore, in relation to nutrition and metabolism, feed and forage toxicants or pollutants, derived primarily from anthropogenic activities, can affect reproduction (McEvoy *et al*., 2001; Rhind, 2008). Logically, these latter factors could also be affecting the early foetal period (McEvoy *et al*., 2001). Similarly, micronutrients can play an important role on pregnancy maintenance; for example, an association between lower zinc and higher serum copper with foetal loss was found in dairy cows (Graham *et al*., 1994).

Genetics is controlling almost every aspect of living organisms, so we have to suppose that genetic parameters could also be affecting early foetal loss. However, the low rate of early foetal loss found in heifers (2.6 %; Chapter 4) is probably indicating us that genetic selection orientated towards milk production is not clearly associated with the increase in rates of early foetal loss observed in the dairy industry. Even so, early studies found greater rates of pregnancy loss in the systems of mating in which that dam was inbred than those in which the dam was outbred (Mares *et al*., 1961), indicating than inbreeding can affect early foetal loss, probably as a consequence of genetic base. Moreover, the fact that semen-providing bulls are affecting early foetal loss rates (Chapter 4; Markusfeld-Nir, 1997) indicates again that probably some genetic traits affect foetal loss. Although little progress has been made on the identification of major genes affecting reproduction traits (Veerkamp and Beerda, 2007), development in genomics, proteomics and bioinformatics would probably identify genetic factors associated with embryo and foetal survival.

Understanding epigenetic mechanisms, which are likely involved in the developmental origin of health and disease (Waterland and Michels, 2007), can also provide some explanations on how nutrition and environmental stimuli influence developmental pathways and induce changes in metabolism, which may be affecting reproductive traits and also conceptus development. In a recent study (Bressan *et al*., 2009), it was proposed that genomic imprinting, an epigenetic phenomenon, may be one of the most important regulatory pathways involved in the development and function of the placenta in eutherian mammals. Thus, a lack of pattern or an imprecise pattern of genomic imprinting can lead to either embryonic losses or a disruption in foetal and placental development.

Chapter 4 represents, in some way, a summary of previous work done in North-eastern Spain. One could argue that validity of results is restricted to the area of the study. As stated above, the reason of grouping results only from that region was that data derived from the same reproductive control program, mimicking an extensive trial. Moreover, although early foetal loss has not been analysed frequently and methods of measuring and definitions are not consistent across all literature, effort has been made to compare results to others obtained elsewhere. Thus, we may expect that results can be valuable for similar production systems of high-producing dairy cows from elsewhere. Even so, increasing research on dairy cattle fertility, especially in the USA, is starting to include early foetal loss as an additional variable of study. Although more research will still provide further understanding of early foetal loss causality, it would be very valuable to put into practice a systematic review and meta-analysis on factors affecting early foetal loss (Blettner *et al.*, 1999; Berman and Parker, 2002; Egger *et al.*, 2002; Bigby and Williams, 2003; Akobeng, 2005). These procedures allow gathering data from different studies quantitatively and re-analyse them using established statistical methods (Petitti, 2000; Sutton, 2000; Pai *et al.*, 2004). Using an analogy currently used in literature on meta-analysis, it is probably time of *mixing apples and oranges*, in order to further elucidate factors affecting non-infectious early pregnancy loss.

9.2 *The hypothesis for therapeutic approaches*

Besides the multifactorial nature of early foetal loss, we hypothesised that one underlying causes of increasing rates noted in high-production dairy cows could be decreased concentrations of peripheral concentrations of progesterone. This has been the foundation for the therapeutic approach proposed in the present thesis. The fact that the only factor favouring the maintenance of gestation was the presence of an additional corpus luteum supports this hypothesis, suggesting that the increase of progesterone levels during the late embryonic period could protect against pregnancy loss. There is no doubt that the corpus luteum is the major source of progesterone in the cow during the first 200 days of gestation (Sawyer, 1995; Niswender *et al.*, 2000). However, many factors are involved in the formation and regulation of luteal function (Niswender *et al.*, 2000; Schams and Berisha, 2004) and some studies showed similar plasma progesterone concentrations for animals with single or two corpora lutea (Starbuck *et al.*, 2004; Mann *et al.*, 2007). In the second work (Chapter 5) we studied some factors affecting progesterone concentrations at the onset of the foetal period and confirmed that, although plasma progesterone concentrations were highly variable among cows, compared to cows with a single corpus luteum, cows with two or more corpora lutea were 3 times more likely to have high plasma progesterone concentrations. Moreover, cows with additional corpora lutea also showed higher plasma progesterone concentrations between day 28 and 34 of gestation (Chapter 6).

Elevated metabolic activity in lactating dairy cow involving an enhanced steroid metabolism seems to be the reason of sub-optimal concentrations of progesterone (Wiltbank *et al*., 2006; Rhinehart *et al*., 2009). Nevertheless, an apparent contradiction rises: if increased milk production involves enhanced steroid metabolism, this should be translated in increased rates of early foetal loss in higher producers; but from the analysis of factors, milk production results a factor with no effect on early foetal loss (Chapter 4). This finding agrees with several studies where there is little or no indication that milk production is a risk factor for increased pregnancy loss in dairy cattle (Chebel *et al*., 2004; Santos *et al*., 2004a,b; Jousan *et al*. 2005; McDougall *et al*. 2005). However, we could found increased early foetal loss in high producers in one study (Bech-Sabat *et al*., 2007). Furthermore, it has been shown that milk production is negatively related to plasma progesterone concentrations at the onset of the foetal period (Rhinehart *et al*., 2009). These results were partly confirmed by this thesis, where this correlation was found during the warm season (Chapter 5).

Although a direct relationship milk production – progesterone concentrations – early foetal loss is difficult to draw, we think this fact does not invalidate directly our standing hypothesis. We recall again to the multifactorial nature of early foetal loss and also of milk production. It has been suggested that approximately half of the progress in milk yield can be attributed to genetics and the remaining half can be attributed to improved environmental factors such as better nutrition, health and management (Pryce *et al*., 2004). Probably high milk production of a specific cow is the result of higher genetic merit, better nutrition and health and higher social status within a particular farm setting. Thus, some subclinical disease or stress situation, for example, could be linked not only to lower production, but also to increased early foetal loss rate. A paradigmatic situation of this is found in a recent study (Silva-Del-Rio *et al*. 2009), where early foetal loss was increased not only in higher producers but also in lower producers, compared with average producing cows.

9.3 *Therapeutic approaches*

Therapeutic approaches included in this thesis were based on strategies: (1) inducing the formation of an additional corpus luteum, or (2) supplying its function using exogenous progestagens, strategies that should help to favour the maintenance of pregnancy in high-producing dairy cows.

9.3.1 Interventions during late embryonic period

Previous studies have shown that progesterone supplementation during the late embryonic period by a progesterone releasing intravaginal device for 28 days reduces early foetal loss either starting between 36-42 days (López-Gatius *et al.*, 2002) or between 28-35 (Bech-Sabat *et al.*, 2007) and also by 1-week supplementation from day 28 to 35 of gestation (Alnimer and Lubbadeh, 2008). On the other hand, the use of gonadotrophins, such as hCG or GnRH, at pregnancy diagnosis, which clearly increased the number of additional corpora lutea, could not reduce the incidence of early foetal loss (Bartolome *et al.*, 2006; Stevenson *et al.*, 2008). Similarly GnRH administration on day 21 or 23 of gestation included in resynchronization protocols had no effect on subsequent pregnancy losses in cows that were pregnant at the time of treatment (Chebel *et al.*, 2003; Bartolome *et al.*, 2005).

In the third study we compared these two therapeutic approaches (Chapter 6). Measuring plasma progesterone concentrations at the time of treatment (at pregnancy diagnosis, days 28-34 of gestation) and seven days later, we could found that while intravaginal progesterone supplementation led to increased plasma progesterone concentrations, GnRH treatment did not. This could partly explain previous results. However, the second experiment of this study (Chapter 6), where early foetal loss rates following these two treatments were evaluated, led us to unexpected results: in cows with a single corpus luteum, the probability of early foetal loss decreased by a factor of 0.51 in cows supplemented with progesterone compared to cows treated with GnRH (accordingly to previous results); but, in cows with two or more corpora lutea, progesterone treatment increased the likelihood of pregnancy loss by a factor of three, compared to GnRH treatment. Perhaps excessive progesterone concentrations impaired embryo-maternal communication in these cows, as gestation is the outcome of a delicate balance among numerous molecules (Wolf *et al.*, 2003; Spencer *et al.*, 2004; Spencer and Bazer, 2004). An alternative explanation is that GnRH treatment mediated the promotion of pregnancy maintenance in cows suffering conceptus reduction. Unfortunately, the experiment was developed in commercial conditions and that fact did not allow us to include a control group of untreated cows. This would have provided a better understanding of how treatments are affecting pregnancy maintenance.

9.3.2 Treatments around AI or during the early embryonic period

We have also evaluated the same therapeutic approaches administrated at earlier stages of pregnancy or even before AI on subsequent early foetal loss, besides of given when pregnancy is confirmed (Chapters 7 and 8). Similarly as when administered during late embryonic period, gonadotrophins at AI or during early embryonic period increased number of additional corpora lutea, but could not reduce subsequent early foetal loss (López-Gatius *et al.*, 2006; Sterry *et al.*, 2006; Stevenson *et al.*, 2007). In the case of progesterone supplementation, literature shows inconclusive results. Whereas some studies could find a positive effect on previous exposure to progesterone on subsequent foetal survival (El-Zarkouny *et* *al*., 2004; Villarroel *et al*., 2004) others could not (Galvao *et al*., 2004; Rivera *et al*., 2005; Sterry *et al*., 2006; Stevenson *et al*., 2006; Cerri *et al*., 2009).

In the fourth work (Chapter 7) we studied if synchronization protocols based on progesterone or progesterone plus GnRH could help to maintain pregnancy during subsequent early foetal period. We observed that oestrous synchronization with progesterone was linked to a reduction of pregnancy loss by a factor of 0.11, whereas progesterone-GnRH combination had not a clear effect (Chapter 7). Although the mechanism remains to be established, the strong effect of progesterone treatment previous to gestation on subsequent early foetal loss supports the hypothesis of decreased concentrations of progesterone in high-producing dairy cows. It also suggests that progesterone priming before AI is important for sequentially programming the brain, the oviduct and uterus with the appropriate changes for future conceptus development. Circulating concentrations of progesterone during the cycle immediately prior to insemination have been related to embryo survival (Diskin *et al*., 2006) and cows cyclic before first postpartum AI had reduced risk of pregnancy loss than anovular cows (Santos *et al*., 2009).

Although fewer losses were recorded in the progesterone-GnRH group than control group, this difference was not significant. A possible explanation is the antagonic anti- (progesterone) and pro-LH (GnRH) effects (Chenault *et al*., 1990; Adams *et al*., 1992) that may create a conflict in the follicular wave and embryo development could be affected as a consequence.

9.4 Induced Corpora Lutea

Despite the presence of spontaneus additional corpus luteum is a strong preventive factor for early foetal loss, gonadotrophin treatments do not seem to promote pregnancy maintenance significantly. In the case of treatments at pregnancy diagnosis, induced corpora lutea may not be fully functional when early foetal loss occurs (Chapter 6). More intriguing is why treatments at early stages of pregnancy which increase the number of pregnancies with additional corpora lutea do not reduce significantly early foetal loss. This fact prompts the question whether induced corpora lutea are fully functional or not.

In humans, the luteal phase has been found to be defective in virtually all the stimulation protocols used in in-vitro fertilization (Lenz and Lindenberg 1990; Tavaniotou *et al*., 2002) and corpus luteum insufficiency is well established after the use of GnRH-agonists (Smitz *et al*., 1988). In cattle, estrous cycle hCG-induced corpora lutea derived from first-wave follicles appeared to be smaller and secrete less progesterone than similar spontaneus corpora lutea of similar age (Sianangama and Rajamahendran, 1996). Abnormal luteal function has also been described after gonadotrophin stimulation in ewes (Southee *et al*., 1988; Hunter *et al*. 1988).

The corpus luteum is a unique transient reproductive gland with maternal instincts but also suicidal tendencies (Davis and Rueda, 2002; López-Gatius et al., 2010), partly auto-regulated by playing the role of being its own judge, jury and executioner (Niswender *et al.*, 2003). Many of the regulatory mechanisms involved in loss of function and involution of the structure are incompletely understood and we are far from understanding how these complex mechanisms function in unison.

Moreover, it has also been found that GnRH can act directly on the corpus luteum, leading to suppressed production and release of progesterone due to inhibitory effect on the cholesterol transport and/or on the enzymes involved in the steroidogenic pathway (Sridaran *et al.*, 1999). GnRH receptors have been demonstated in the bovine ovary (Ramkrishnappa *et al*. 2003) and also in other bovine reproductive tissues (Singh *et al*., 2008).

All these facts clearly open new research lines for understanding better both corpus luteum functionality and gonadotrophin treatments.

9.5 *Further prospects*

The epidemiological approach has permitted to define and quantify the clinical picture on early foetal loss and develop strategies for intervention. The incorporation of other variables on the surveillance system can provide new information for designing different strategies to reduce the incidence of early foetal loss. Furthermore, new studies on the underlying physiological mechanisms of the factors affecting early foetal loss are also required.

The incorporation of colour Doppler processing of genital blood flow to transrectal ultrasonography has extended the scope of ultrasound images to provide physiologic as well as anatomic information (Ginther, 1995; Herzog and Bollwein, 2007; Matsui and Miyamoto, 2009). Blood flow in the corpus luteum correlates better with progesterone secretion than does size of corpus luteum (Herzog and Bollwein, 2007). Specific therapies applied only to cows suffering anomalous function of the corpus luteum, as diagnosed by blood flow ultrasonography, should improve both the treatment response compared with treatment applied to all animals and our understanding of foetal loss related to ovarian function (López-Gatius *et al*., 2010).

Not only the ovarian function is essential for a successful pregnancy, but also the uterine function. Detailed studies of the uterus should contribute to understand the underlying causes of pregnancy loss. Previous placenta or pyometra resulted in increased risk of subsequent early foetal losses (Chapter 4). It is possible that these disorders caused alterations in the uterine environment, which could be particularly critical during the implantational process when maternal-conceptus interactions are in progress and trophoblast is attaching to the uterine mucosa and becoming dependent of the placentomes. Traces of uterine disease could interfere with the course of attachment; this in turn could in part explain why non-parous heifers had a decreased risk of early foetal loss, since they are less likely to develop uterine disease. Moreover, it is important to consider that hormones, and other molecules, act through its receptors. Thus, it is important not only concentrations of hormones, but also the presence of adequate number of receptors. A better understanding of uterine and placental receptivity to hormones should clarify the action of progesterone before AI or during early embryonic period on subsequent pregnancy loss.

As commented in Chapter 8, other therapeutic approach would be the use of methods to decrease excessive hepatic clearance of steroid hormones without compromising dry matter intake and other metabolic functions. Decreasing the expression of progesterone catabolic enzymes during early pregnancy, and thereby extending the biological half-life of steroid hormones, may be a useful approach for increasing peripheral concentrations of progesterone (Lemley *et al*., 2008). Feeding diets that stimulate insulin secretion could alter progesterone clearance during lactation (Smith *et al*., 2006; Lemley *et al*., 2010). Therefore, introducing dietary inputs which are able to decrease progesterone clearance may provide interesting strategies for reducing early foetal loss. This is an interesting approach, especially if we consider the growing concern of consumers and law-makers on the use of hormones (Refsdal, 2000).

Twin pregnancies are undesirable in dairy cattle since besides increasing the risk of early pregnancy loss, they also have negative effects such as increased abortion, dystocia, retained placenta, calf mortality, occurrence of freemartins and risk of premature culling, longer postpartum therapy and calving to conception intervals, and less milk production after calving (Nielen *et al*., 1989; Gregory *et al*., 1996; Bicalho *et al*., 2007). When assessed, this represents a great economic impact (Eddy *et al*., 1991; Beerepoot *et al*., 1992). Despite the success of GnRH treatment for twin pregnancies noted above (Chapter 7), such negative effects of twinning might be diminished by reducing the embryo number in dairy cows. Embryo reduction methods have been used in potentially dangerous multiple pregnancies in humans (Mansour *et al*., 1999; Iberico *et al*., 2000) and in the treatment of twin pregnancies in mares (Macpherson and Reimer, 2000; Mckinnon, 2007; Ragon, 2007). In fact, the procedure of rupturing an embryo vesicle before fixation has become a common strategy among equine veterinary practitioners (Frazer, 2003) with great success (Macpherson and Reimer, 2000). In cows, the procedure of rupturing the amnion plus progesterone supplementation has been found to provide an open way for twin reduction in dairy cattle (López-Gatius, 2005). Transvaginal ultrasound-guided embryo aspiration could be an alternative to amnion rupture for reduction of twin pregnancy in the cow, as it has been also proposed in the mare to reduce twins after fixation (Bracher *et al*., 1993; Macpherson and Reimer, 2000; Mari *et al*., 2004) and in humans (Coffler *et al*., 1999). Ecoguided transvaginal amnion and alantocentesis techniques have been already developed in the cow, although with different purposes than embryo reduction and not without some risk for conceptus viability (Vos *et al*., 1990; Garcia and Salaheddine, 1997; Kamimura *et al*., 1997; Makondo *et al*., 1997).

9.5 References

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10

Conclusions

10 CONCLUSIONS

In high producing dairy cows and under our working conditions, a summarised list of conclusions obtained from the studies derived of this thesis includes:

1. Factors strongly affecting late embryonic and early foetal loss were parity (lactating cows versus heifers), semen-providing bull, warm season and twin pregnancies, whereas the presence of an additional corpus luteum was identified as a strong positive factor favouring the maintenance of gestation.

2. Low milk production during the warm season and the presence of two or more corpora lutea were related to increased plasma progesterone at the onset of the early foetal period.

3. A positive association was drawn between high concentrations of progesterone and high concentrations of PAG-1 in primiparous cows at the onset of the early foetal period.

4. Progesterone supplementation at pregnancy diagnosis led to a significant increase in plasma progesterone concentrations 7 days after starting treatment, whereas similar progesterone levels were recorded after 7 days of GnRH treated and untreated control cows.

5. In cows carrying singletons with a single corpus luteum, progesterone supplementation halved the likelihood of early foetal loss compared to cows receiving GnRH.

6. In cows with two or more corpora lutea, progesterone supplementation triplicated the likelihood of pregnancy loss compared to cows receiving GnRH.

7. The conceptus reduction rate in cows carrying twins was significantly higher after GnRH treatment than after progesterone supplementation at pregnancy diagnosis.

8. Although a progesterone-based oestrous synchronization protocol in 51- to 57-day postpartum did not improve conception rate at first AI, treated cows could be inseminated earlier than untreated cows such that by day 120 postpartum cumulative conception rates were higher.

9. The likelihood of early foetal loss following first insemination was 0.11 times lower in cows treated with a progesterone-based oestrous synchronization protocol compared with control cows.

10. Possible benefits of gonadotrophins or progesterone in the period before AI or during early embryonic period in terms of reducing subsequent early foetal loss are not clear.

11. Adequate therapeutic interventions at pregnancy diagnosis require to record the number of corpora lutea and the number of embryos.

12. Treatment for reducing early foetal loss is not necessary in cows with an additional corpus luteum.

Summary/Resum/Resumen

SUMMARY

Pregnancy failure is one of the single most economically important problems faced by the modern dairy industry, with a great effect if losses occur between 30 and 60 days of gestation, during the late embryonic and early foetal periods. Incidence of late embryonic/early foetal loss is increasing under the intensive management systems used today, and rates may exceed 20%. In this thesis we studied this reproductive disorder through an epidemiological approach. Ultrasound and determination of reproductive hormones were the basic research tools. The main aim was to understand which factors are affecting and propose therapeutic approaches to reduce its incidence. Treatments were based on the hypothesis that low levels of progesterone on high producing dairy cows may be involved in the increasing rates of losses. Research included in this thesis was divided in five studies published in peer-reviewed journals.

In the first study we presented data published during the period of 1996 to 2008, analysing the effects of factors of non-infectious nature affecting early foetal loss in high producing dairy herds of north-eastern Spain. Factors strongly affecting early foetal loss were parity (lactating cows vs heifers), semen-providing bull, warm season and twin pregnancies, whereas the presence of an additional corpus luteum was the only factor favouring the maintenance of gestation.

In the second chapter we studied the factors affecting progesterone concentrations at the onset of the foetal period. Cows with two or more corpora lutea were 3 times more likely to have high plasma progesterone concentrations compared to cows with a single corpus luteum and had a strongly diminished risk of early foetal loss. Milk production and PAG-1 concentrations were also affecting progesterone concentrations.

In the third study we compared GnRH and progesterone treatments at pregnancy diagnosis. Progesterone supplementation led to a significant increase in plasma progesterone concentrations whereas GnRH treatment could not. In cows carrying singletons with a single corpus luteum, progesterone supplementation halved the likelihood of early foetal loss compared to cows receiving GnRH. In cows with two or more corpora lutea, progesterone supplementation triplicated the likelihood of pregnancy loss compared to cows receiving GnRH. Moreover, the conceptus reduction rate in cows carrying twins was significantly higher after GnRH treatment.

The aim of the fourth work was to investigate the effects of applying a progesterone-base oestrous synchronization protocol at 51-57 days postpartum in high-producing dairy cows. Oestrous and conception rates before days 71-77 postpartum, pregnancy loss in early pregnant cows and the cumulative conception rate registered on day 120 postpartum were considered as dependent variables in four consecutive logistic regression analyses. Although progesterone-based

oestrous synchronization protocols in 51- to 57-day postpartum did not improve conception rate at first AI, treated cows could be inseminated earlier than untreated cows such that by day 120 postpartum cumulative conception rates were higher. Unexpectedly the likelihood of early foetal loss following first insemination was 0.11 times lower in cows treated with a progesterone-based oestrous synchronization protocol compared with control cows.

The fifth work was a review of recent therapeutic approaches designed to reduce the incidence of early foetal loss. Progesterone supplementation during the late embryonic and early foetal period was found to be useful in curtailing losses in single pregnancies. Therapeutics is not necessary in cows with additional corpus luteum. Possible benefits of gonadotrophins or progesterone in the period around AI in terms of reducing subsequent early foetal loss were not clear in the literature. Large trials or meta-analytic procedures could clarify this issue.

RESUM *(Català)*

La pèrdua de gestació és un dels problemes més importants que ha d'encarar la indústria lletera moderna, amb un efecte major si les pèrdues ocorren entre 30 i 60 dies de gestació, durant el període embrionari tardà i fetal primerenc. La incidència de pèrdua fetal primerenca està incrementant en els sistemes intensius de producció lletera que s'usen avui, amb taxes que poden superar el 20 %. En aquesta tesi, s'ha estudiat aquest desordre reproductiu a través d'un acostament epidemiològic. L'ecografia i la determinació d'hormones reproductives han estat les principals eines de recerca. El principal objectiu fou entendre quins factors l'afecten i proposar un acostament terapèutic, basant-nos en la hipòtesi que els baixos nivells de progesterona de les vaques lleteres d'alta producció augmenten les pèrdues de gestació en el període embrionari tardà i fetal primerenc. El treball inclòs en aquesta tesi fou dividit en 5 capítols, els qual s'han publicat en revistes científiques.

En el primer estudi es presenten dades publicades en el període 1996-2008, analitzant els efectes de factors de naturalesa no infecciosa que afecten la pèrdua fetal primerenca en vaques lleteres d'alta producció de la zona de Lleida-Osca. Els factors que resultaren afectar fortament a la pèrdua fetal primerenca foren la paritat (vaques lactants vs vedelles), el toro, l'estació calorosa i les bessonades mentre que la presència d'un cos luti addicional fou l'únic factor identificat com afavoridor del manteniment de la gestació.

En el segon capítol s'estudiaren els factors que afecten a les concentracions de progesterona a l'inici del període fetal. Vaques amb múltiples cossos lutis tingueren 3 vegades més probabilitats de tenir altes concentracions de progesterona, comparat amb les vaques amb 1 sol cos luti i veieren fortament disminuïda la probabilitat de perdre la gestació. La producció lletera i els nivell de PAG-1 també es relacionaren amb les concentracions de progesterona.

En el tercer estudi es compararen els tractaments amb GnRH i progesterona al moment del diagnòstic de gestació. La suplementació amb progesterona portà a un increment significatiu de les concentracions plasmàtiques de progesterona, mentre que el GnRH no en fou capaç. En vaques amb 2 o més cossos lutis, la suplementació amb progesterona triplicà la probabilitat de pèrdua comparant amb les que reberen GnRH. A més, la taxa de reducció embrionària en bessonades fou significativament més alta després del tractament amb GnRH.

L'objectiu del quart estudi fou la investigació dels efectes de l'aplicació els dies 51 – 57 postpart de protocols de sincronització de l'estre basats en progesterona en vaques d'alta producció. La taxa d'estre i de concepció a dia 71 – 77 postpart, la pèrdua de gestació primerenca i la taxa de concepció acumulada a dia 120 postpart foren considerades com a variables dependents en quatre anàlisis de regressió logística consecutius. Tot i que els protocols de sincronització no pogueren millorar

la taxa de concepció a la primera inseminació, les vaques sincronitzades pogueren ser inseminades abans que les no tractades i pel dia 120 la taxa de concepció acumulada fou més alta. Inesperadament, la probabilitat de pèrdua de gestació primerenca després dels protocols de sincronització basats en progesterona fou 0.11 vegades respecte la taxa de pèrdua de les vaques control.

El cinquè treball fou una revisió dels diferents acostaments terapèutics designats per a reduir la pèrdua fetal primerenca. La suplementació amb progesterona durant el període embrionari tardà i fetal primerenc s'ha trobat útil per a reduir les pèrdues del primer trimestre. La terapèutica no resulta necessària en vaques amb cos luti addicional. Els possibles beneficis del tractament amb gonadotropines o progesterona en el període abans de la inseminació o durant l'etapa embrionària primerenca, en termes de reduir les pèrdues fetals primerenques posteriors, no resultà clar a la literatura. Farien falta experiments més extensius o procediments meta-analítics per a clarificar aquesta qüestió.

RESUMEN *(Castellano)*

La pérdida de gestación es uno de los problemas más importantes que tiene que afrontar la industria lechera moderna, con un mayor efecto si éstas ocurren entre los días 30 y 60 de gestación, durante el periodo embrionario tardío y fetal temprano. La incidencia de pérdidas fetales tempranas está aumentando en los sistemas intensivos de producción lechera actuales, con tasas que pueden superar el 20 %. En esta tesis, se estudió este desorden reproductivo a través de un enfoque epidemiológico. La ecografía y la determinación de hormonas reproductivas fueron las principales herramientas para su estudio. El principal objetivo fue entender que factores la afectan y proponer un acercamiento terapéutico, basándonos en la hipótesis que los bajos niveles de progesterona de las vacas lecheras de alta producción se relacionan con un aumento de pérdidas de gestación durante el periodo embrionario tardío y fetal temprano. El trabajo incluido en esta tesis fue dividido en 5 capítulos, publicados en revistas científicas.

En el primer estudio se presentan datos publicados en el período 1996-2008, analizando los efectos de factores de naturaleza no infecciosa que afectan la pérdida fetal temprana en vacas lecheras de alta producción de la zona de Lérida-Huesca. Los factores que resultaron afectar fuertemente a la pérdida fetal temprana fueron la paridad (vacas lactantes frente novillas), el toro, la estación cálida i las gestaciones gemelares, mientras que la presencia de un cuerpo lúteo adicional fue el único factor identificado como favorecedor del mantenimiento de la gestación.

En el segundo capítulo se estudiaron factores que afectan a las concentraciones de progesterona en el inicio del período fetal. Las vacas con múltiples cuerpos lúteos tenían 3 veces más probabilidades de tener altas concentraciones de progesterona, comparado con las vacas con 1 solo cuerpo lúteo, además de verse fuertemente reducidas sus probabilidades de perder la gestación. La producción lechera y los niveles de PAG-1 también se relacionaron con las concentraciones de progesterona.

En el tercer estudio se compararon los tratamientos con GnRH y progesterona en el momento del diagnóstico de gestación. La suplementación con progesterona supuso un incremento significativo de las concentraciones plasmáticas de progesterona, mientras que el tratamiento con GnRH no. En vacas con 2 o más cuerpos lúteos, la suplementación con progesterona triplicó la probabilidad de pérdida comparado con las que recibieron GnRH. Además, la tasa de reducción embrionaria en gestaciones gemelares fue significativamente más alta después del tratamiento con GnRH.

El objetivo del cuarto estudio fue la investigación de los efectos de la aplicación los días 51 – 57 posparto de protocolos de sincronización del estro a base de progesterona en vacas de alta producción. La tasa de estro y de concepción a día 71 – 77 posparto, la pérdida de gestación temprana y la tasa de concepción acumulada a día 120 posparto fueron consideradas como variables dependientes en cuatro análisis de regresión logística consecutivos. Aunque los protocolos de sincronización no mejoraron las tasas de concepción a primera inseminación, las vacas sincronizadas pudieron ser inseminadas antes que las no tratadas y la tasa de concepción acumulada a día 120 fue superior. Inesperadamente, la probabilidad de pérdida de gestación temprana después de los protocolos de sincronización a base de progesterona fue 0.11 veces respeto la tasa de pérdida en vacas control.

El quinto trabajo fue una revisión de los diferentes acercamientos terapéuticos diseñados para reducir la pérdida fetal temprana. La suplementación con progesterona durante el periodo embrionaria tardío y fetal temprano ha resultado útil para reducir las pérdidas del primer trimestre. La terapéutica no es necesaria en vacas con cuerpo lúteo adicional. Los posibles beneficios del tratamiento con gonadotropinas o progesterona en el periodo antes de la inseminación o durante el período embrionario temprano, en términos de reducir las pérdidas fetales tempranas subsiguientes, no resultaron claros en la literatura. Experimentos más extensivos o procedimientos meta-analíticos serian necesarios para clarificar esta cuestión.

Agraïments (Acknowledgments)

Appendix

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AGRAÏMENTS (ACKNOWLEDGEMENTS)

La tesi doctoral representa el final d'un etapa. Una etapa que vaig començar ja fa més de 4 anys. No és el resum de tot aquest temps, però segurament sí que n'és, d'alguna manera, la culminació. Per això els agraïments haurien d'incloure tota la gent que d'una manera o altra s'ha creuat amb mi i s'ha interessat pel què he fet, m'ha ajudat, recolzat, animat i cregut en mi durant aquest llarg període. No em veig capaç d'incloure'ls a tots aquí; segurament tampoc és el lloc per fer-ho. Espero haver sabut donar les gràcies quan ha sigut el moment. Tot i això, no puc deixar passar aquest moment per reiterar explícitament el meu agraïment a algunes de les persones que han fet possible que l'etapa doctoral m'hagi permès escriure aquesta tesi, i més important, m'hagi format en tants sentits.

No tinc cap dubte en que qui més ha contribuït a aquesta tesi, i a tot el què representa, és el meu director de tesi, en Fernando. Poder-lo tenir com a mentor, captar la seva forma de veure el món i haver pogut travar amistat amb ell, ha estat un gran privilegi. Dit amb una frase pròpia d'ell, dificilment mai podré sobrevalorar-ho.

De la mateixa manera, vull agrair al meu co-director, en Jesús. El seu suport durant aquests anys i l'empenta que té han estat de gran valor i un exemple del que aprendre molt. La seva proximitat en tot moment i especialment en l'elaboració del document de tesi ha estat fonamental. Tenim molts motius de futures col·laboracions, que espero no deixem passar.

També vull fer explícits els agraïments a la resta del grup d'investigació. Un grup, que tinc la sensació d'haver vist créixer de forma espectacular. La Irina ha estat una peça clau i veure el seu exemple ha sigut una gran sort. La Pilar, la Bea, la Carmina i la Paqui fan del grup una cosa molt agradable i totes han participat també en la meva formació. Durant l'últim període, ha sigut una grandíssima experiència poder gaudir de companys de tesi com la Cris, la Irene i l'Ahmed. També voldria agrair a la part no-directament investigadora, però fonamental del grup, que són les granjes en les que treballem, sobretot a la Granja Allué i la de l'Óscar Marí, i també a la San José, l'Aníbal i el Palau. No em puc deixar la resta de gent del departament de producció animal i de l'escola que també han estat presents durant aquests anys, especialment en Xavi i l'Assu, companys de tesi fonamentals.

He tingut la sort de poder moure'm bastant durant aquest temps, cosa que sens dubte enriqueix l'experiència doctoral de forma significativa. Gràcies a tota la gent del curs de l'IAMZ de Zaragoza, especialment a en Javi, i la resta amb qui vaig conviure durant aquesta fase. També a tothom amb qui vaig compartir l'estada a Liège al laboratori del professor Beckers i la Noelita, i l'estada a Madrid dins el grup SALUVET, dirigit per en Luis Ortega. Sentir-se tan acollit a Liège i a Madrid ha estat una grandíssima experiència.

No sé si té molt sentit agrair a un conjunt de gent que ni conec, i que part ja no hi és, però sí voldria fer menció a un conjunt de gent que són segurament els que més m´han acompanyat durant aquests anys: tota la gent que fa Música, la gran passió de la meva vida. I la gran inspiradora.

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A tots, i tots els que no estan escrits aquí però llegiu això (prova que també em recolzeu, us interesseu, o m'heu ajudat en aquest camí doctoral), moltes gràcies.

Lleida, Juny 2010

ON THE PUBLICATIONS INCLUDED IN THE PRESENT THESIS

Chapters 4 to 8 of the present thesis have been previously published as articles in peer-reviewed journals. Information on the publications is included in this section.

Chapter 4

Original publication: Factors of non-infectious nature affecting late embryonic and early foetal loss in high producing dairy herds in north-eastern Spain. Literature review.

Authors (in order of authorship): F. López-Gatius[1], O. Szenci[2], G. Bech-Sàbat[1], I. García-Ispierto[1], B. Serrano[1], P. Santolaria[3], J. Yániz[3]
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Date of Publication: September 2009

Reference: Magyar Állatorvosok Lapja 131:515-531
a) Indexation Data Base: Science Citation Index (SCI)
b) Latest impact factor (2009): 0.200
c) Subject and Position within it (by impact factor): VETERINARY SCIENCES: 115 out of 141

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Chapter 5

Original publication: Factors affecting plasma progesterone in the early fetal period in high producing dairy cows. *Theriogenology* 69:426-432.

Authors (in order of authorship): G. Bech-Sàbat[1], F. López-Gatius[1], J.L. Yániz[2], I. García-Ispierto[3], P. Santolaria[2], B. Serrano[4], J. Sulon[5], N.M. de Sousa[5], J.F. Beckers[5]
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Date of publication: March 2008 (Published on-line 28 November 2007, doi:10.1016/j.theriogenology.2007.10.012)

Reference: Theriogenology 69:426-432
a) Indexation Data Base: Science Citation Index (SCI)
b) Latest impact factor (2009): 2.073
c) Subject and Position within it (by impact factor): VETERINARY SCIENCES: 12 out of 141; REPRODUCTIVE BIOLOGY: 16 out of 26

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Chapter 6

Original publication: Pregnancy patterns during the early fetal period in high producing dairy cows treated with GnRH or progesterone.

Authors (in order of authorship): G. Bech-Sàbat[1], F. López-Gatius[1], I. García-Ispierto[1], P. Santolaria[2], B. Serrano[1], C. Nogareda[1], N.M. de Sousa[3], J.F. Beckers[3], J.L. Yániz[2]
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Date of publication: April 2009 (Published on-line 19 December 2008; doi:10.1016/j.theriogenology.2008.10.013)

Reference: Theriogenology 71:920-929
a) Indexation Data Base: Science Citation Index (SCI)
b) Latest impact factor (2009): 2.073
c) Subject and Position within it (by impact factor): VETERINARY SCIENCES: 12 out of 141; REPRODUCTIVE BIOLOGY: 16 out of 26

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Chapter 7

Original publication: Effects of a Progesterone-Based Oestrous Synchronization Protocol in 51- to 57-Day Postpartum High-Producing Dairy Cows. Reproduction in Domestic Animals

Authors (in order of authorship): I. García-Ispierto[1], F. López-Gatius[1], G. Bech-Sàbat[1], J. Yániz[2], E. Angulo[1], C. Maris[1], S. Floc'h[3], A. Martino[4]
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Date of Publication: In press (Published on-line: 18 November 2009, doi: 10.1111/j.1439-0531.2009.01541.x)

Reference: Reproduction in Domestic Animals, *in press*
a) Indexation Data Base: Science Citation Index (SCI)
b) Latest impact factor (2009): 1.606
c) Subject and Position within it (by impact factor): VETERINARY SCIENCES: 25 out of 141; REPRODUCTIVE BIOLOGY: 19 out of 26; AGRICULTURE, DAIRY & ANIMAL SCIENCE: 11 out of 49.

Acknowledgements: The authors thank Ana Burton for assistance with the English translation and Paqui Homar for help with the data collection. This work was supported by the Spanish CICYT, grants AGL2007-65521-C02-01/GAN, AGL2007-65521-C02-02/GAN. Bech-Sàbat was supported by an FPU grant from the Ministerio de Educación y Ciencia, AP-2005-5378.

Chapter 8

Original publication: Therapeutic Approaches to Pregnancy Loss of Non-infectious Cause During the Late Embryonic/Early Foetal Period in Dairy Cattle. A Review.

Authors (in order of authorship): G. Bech-Sàbat[1], I. García-Ispierto[1], J. Yániz[2], F. López-Gatius[1]
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Date of publication: In press (Published on-line 23 December 2009, doi: 10.1111/j.1439-0531.2009.01562.x)

Reference: Reproduction in Domestic Animals, *in press*
a) Indexation Data Base: Science Citation Index (SCI)
b) Latest impact factor (2009): 1.606
c) Subject and Position within it (by impact factor): VETERINARY SCIENCES: 25 out of 141; REPRODUCTIVE BIOLOGY: 19 out of 26; AGRICULTURE, DAIRY & ANIMAL SCIENCE: 11 out of 49.

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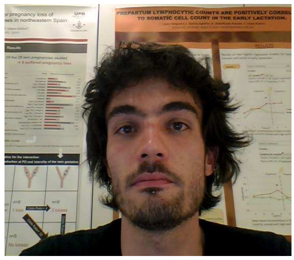

Gregori Bech Sàbat was born in Peralada (Girona, Catalonia, Spain) on October 22nd 1981. He studied the 5-year Agricultural Engineering Degree at the University of Lleida (Catalonia, Spain), focusing his studies on Animal Husbandry. He participated in a 1-year ex-change program at Wageningen University (The Netherlands) within Animal Production Systems and Biological Farming Systems departments.

After collaborating with the veterinary company CEVA Salud Animal from November 2005 till February 2006, he started his PhD at the University of Lleida, under "*Sistemes Agrícoles, Forestals i Alimentaris*" program. He took the required courses at the IAMZ-CIHEAM center of Zaragoza (Spain), doing the Reproduction part of an internationally recognized Animal Production Master.

His PhD has been developed in the research group "Fertility and Preganancy Maintenance in High-Producing Dairy Cows" directed by Fernando López Gatius. Main research lines of the group are the epidemiology of fertility and pregnancy loss in cattle, bovine neosporosis and different aspects of reproductive physiology of domestic animals. A list of publications derived from research he has participated is listed below. During his PhD, he participated in several international and national congresses. He enjoyed a 4-month stay at the Laboratory of Endocrinology of the Unversity of Liège (Belgium), directed by Jean-François Beckers and Noelita de Sousa and another 4-month stay at SALUVET group in the Animal Health Department of the Complutense University of Madrid (Spain), directed by Luis Ortega Mora. He also participated as a lecturer at the University of Lleida in courses of Animal Physiology, Biotechnology of Reproduction and Animal Biotechnology.

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