EARLY PREGNANCY DIAGNOSIS AND EMBRYO/FETUS MORTALITY IN CATTLE

A Dissertation

by

JUAN EDUARDO ROMANO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Physiology of Reproduction

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Approved as to style and content by:

Duane C. Kraemer (Co-Chair of Committee)	David W. Forrest (Co-Chair of Committee)
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ABSTRACT

Early Pregnancy Diagnosis and Embryo/Fetus Mortality in Cattle. (December 2004)
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Co-Chairs of Advisory Committee: Dr. Duane C. Kraemer
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Pregnancy diagnosis by transrectal ultrasonography (using a 5 MHZ linear probe) presented the maximum sensitivity and negative predictive values at day 26 and day 29 after estrus in heifers and cows, respectively.

Palpation per rectum using the fetal membrane slip for pregnancy diagnosis did not increase embryo/fetus mortality when compared with a positive control group of non-palpated females. The use of a controlled randomized block design was a useful approach to study this problem. Blocking for category and number of embryos allowed us to remove these confounding factors.

Factors that affected pregnancy loss during the first four months of pregnancy were: period of pregnancy, age of the animal, number of previous lactations and number of embryos. Pregnancy loss was higher during the embryonic than fetal periods. Spontaneous embryo/fetal mortality increased with the age of the animal and lactation number. The risk of spontaneous embryo/fetus mortality was higher in twin than in single pregnancies.

Two types of embryo/fetus mortality were noted: Type I and Type II. Type I was characterized by presence of positive fetal membrane slip by palpation per rectum, signs of degeneration by transrectal ultrasonography and persistence of a functional corpus luteum. The uterus took approximately 3 weeks to be noted clean by transrectal ultrasonography and the animals showed estrus one month after the conceptus was diagnosed dead. Type II was characterized by absence of positive signs of pregnancy by palpation per rectum, absence of signs of degeneration by transrectal ultrasonography and absence of a functional corpus luteum.

Pregnancy loss in nuclear transfer derived embryos was higher compared to *in vivo* derived embryos produced by artificial insemination. Pregnancy loss occurred mainly during the transition from the embryonic to the fetal period. Embryo/fetus mortality detected was Type I. Progesterone produced by the corpus luteum was noted at pregnancy levels for approximately two weeks after embryo/fetus death. Protein B, a hormonal placental marker, was maintained at pregnancy levels for approximately 3 weeks after embryo/fetus death. No differences in the levels of the two hormones were noted when comparing females with dead or live conceptuses.

DEDICATION

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CHAPTER I

INTRODUCTION

TRANSRECTAL ULTRASONOGRAPHY FOR PREGNANCY DIAGNOSIS

An important step in dairy management is to examine for pregnancy within 45 days after breeding (Zemjanis, 1971). The main purpose of examining cows early is not only to identify pregnant cows but also to identify with confidence open cows in order to manage, treat and cull (Zemjanis, 1971). Diagnosis of non-pregnancy prior to the second expected estrus will allow making a management decision before the next estrus (Zemjanis, 1971). Pregnancy diagnosis at a later stage insures loss of an additional 18-24 days if the cow is not pregnant and no estrous synchronization is applied. Thus, an accurate method of determining pregnancy/non-pregnancy before the expected second estrus would be ideal (Studer, 1969; Zemjanis, 1971). Early pregnancy diagnosis can assist dairy producers in managing open cows and improving reproductive performance and economics of their herd (Oltenacu *et al.*, 1990). Early detection of non-pregnancy should lead to earlier intervention and consequently shorter, more economical calving intervals. It was shown that the earlier the pregnancy diagnosis is performed, the more profitable is the return (Oltenacu *et al.*, 1990).

In bovine practice, two methods allow us to immediately diagnose pregnant/non-pregnant females: palpation per rectum and transrectal ultrasonography. Palpation per rectum as a direct method for pregnancy diagnosis is performed 30 days after breeding/artificial insemination/embryo transfer. However, neither critical studies in regard to its accuracy at earlier stages nor comparing it with transrectal ultrasonography are available. Transrectal ultrasonography for pregnancy diagnosis offers some advantages over palpation per rectum: earlier diagnosis of pregnancy/non-pregnancy,

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This dissertation follows the style of *Reproduction*.

determination of embryo/fetus viability, reduction of misdiagnosis (false negatives and false positives) and reduction of "potential" iatrogenic embryo/fetal attrition (Romano and Magee, 2001). There are two main concerns regarding early use of transrectal ultrasonography for pregnancy diagnosis: safety and accuracy. Reports in veterinary medicine have shown transrectal ultrasonography to be a safe technique that did not affect embryo/fetus viability (Kahn, 1992; Ball and Longue, 1994; Baxter and Ward, 1997). In order to increase the accuracy of the non-pregnant diagnosis a procedure with high negative predictive value that eliminates the possibility of false negatives is required. Consequently, a diagnosis of non-pregnancy will be made with confidence and immediate action taken, such as: treatment, estrous synchronization, culling or sale.

The use of new protocols for estrous synchronization with artificial insemination at fixed time (Wiltbank, 1998; Stevenson *et al.,* 2000) requires an early and accurate method of non-pregnancy diagnosis in order to enroll these animals in a new round of estrous synchronization. In embryo transfer programs, the accuracy of pregnancy/non-pregnancy diagnosis will enable open recipients to be returned or excluded from the recipient herd as soon as possible. Some studies recommend the use of transrectal ultrasonography at 25 or 26 days after breeding to determine the pregnancy/non-pregnancy status (Fissore *et al.*, 1986; Pieterse *et al.*, 1990; Lares *et al.*, 2002; Fricke, 2002). However, these studies are in contrast with previous reports (Badtram *et al.*, 1991; Hanzen and Laurent, 1991) and our experience. At 25-26 days post-insemination the sensitivity and negative predictive values reported are far from being 100 % (Badtram *et al.*, 1991; Szenci *et al.*, 1995; Filteau and DesCôteaux, 1998), therefore, the possibility of diagnosing an animal incorrectly as non-pregnant (false negative) is highly probable. At present, these females incorrectly diagnosed as non-pregnant will receive prostaglandin F-2α immediately or will be schedule in one of the new protocols of estrous synchronization at fixed time (that include the administration of prostaglandin F-2α) causing immediate abortion, therefore, these animals will never be detected as being pregnant. The induction of iatrogenic abortion due to misdiagnosis is not acceptable technically nor economically, especially now that fertility in lactating dairy cattle seems

have declined compared to 20-25 years ago (MacMillan *et al.*, 1996; Lucy, 2001). The negative predictive value of an early pregnancy diagnosis should therefore be 100% to avoid inducing early abortion, culling or selling "unnecessarily", pregnant females. Few studies were designed to evaluate the best day of pregnancy diagnosis with the maximum sensitivity and negative predictive value. In our previous experience, under non-controlled situations, heifers were able to be detected earlier than cows with confidence as pregnant/non-pregnant animals. Differences in uterine characteristics as well as in uterine position were probably the factors that allow an earlier detection in heifers. In most of the studies, cows or heifers were used (Kastelic *et al.*, 1989; Willemse and Taverne, 1989; Pieterse *et al.*, 1990; Badtram *et al.*, 1991; Hanzen and Laurent, 1991; Filteau and DesCôteaux; 1998) or both categories were analyzed together (Chaffaux *et al.*, 1986; Hansen and Delsaux, 1987). Few studies comparing cows versus heifers were found (Hughes and Davies, 1989; Badtram *et al.*, 1991; Hanzen and Laurent, 1991). In one report, a negative correlation between age of the female and accuracy of pregnancy diagnosis in females evaluated by transrectal ultrasonography at 4 weeks was noticed (Hughes and Davies, 1989), however, in two further studies no differences were found (Badtram *et al.*, 1991; Hanzen and Laurent, 1991).

PALPATION PER RECTUM FOR PREGNANCY DIAGNOSIS ON EMBRYO/FETUS MORTALITY

The first report of pregnancy diagnosis in cattle by palpation per rectum dated from the early 1800's (Cowie, 1948). Rectal examination for pregnancy diagnosis was later described as a safe method whose risks have been greatly exaggerated (Fleming, 1896). Since then, numerous workers have from time to time stressed the importance of palpation per rectum of the uterus as a routine measure in the diagnosis of pregnancy and sterility (Dalrymple, 1907; Williams, 1921; Hammond, 1927; Burgess, 1942; Asdell, 1955).

Today, palpation per rectum is the most frequent method used for pregnancy diagnosis in cattle after 30 days of breeding/artificial insemination (Roberts, 1971; Momont, 1990; Youngquist, 1997). It is also common practice for bovine practitioners involved in beef cattle management and among those involved in embryo transfer and other reproductive bio-technologies. It is considered that a good practitioner is able to detect pregnant/non-pregnant animals from day 35 on (Euler, 1930; Götze, 1940; Roberts, 1971; Zemjanis, 1971; Momont, 1990). The importance of a systematic and non-traumatic technique of palpation per rectum cannot be overemphasized as it is well known that embryonic/fetal deaths can be induced accidentally or iatrogenically by this procedure (Ball and Carroll, 1963; Rowson and Dott, 1963; Dawson, 1974; Parmigiani *et al.*, 1978).

There is contradictory information about the potential deleterious effect of palpation per rectum for early pregnancy diagnosis on embryo/fetus viability. Some studies have suggested a possible adverse effect of early palpation per rectum (Abbitt *et al.*, 1978; Paisley *et al.*, 1978; Vaillancourt *et al.*, 1979; Franco *et al.*, 1987; White *et al.*, 1989; McLeod and Williams, 1991). In contrast, other recent studies (Thurmond and Picanso, 1993; Thompson *et al.*, 1994) have suggested little effect of the time at which the first palpation per rectum is performed after insemination on calving rate. These studies had several flaws in their design. Previous reports that diagnosed pregnant females by palpation per rectum (Abbitt *et al.*, 1978, Paisley *et al.*, 1978; Vaillancourt *et al.*, 1979), progesterone (Franco *et al.*, 1987) or protein B (Humblot *et al.*, 1988a; Alexander *et al.*, 1995) did not assess the viability of the embryo/fetus. Most of the studies lack a "pregnant non-palpated group" (control group) (Abbitt *et al.*, 1978, Paisley *et al.*, 1978; Vaillancourt *et al.*, 1979) to differentiate the effects of palpation per rectum from spontaneous embryo/fetal death occurring during early pregnancy. The interval between palpation per rectum and reevaluation was variable: from 30 to 90 days (Abbitt *et al.*, 1978), 44 to 48 days (Franco *et al.*, 1987), or at calving (Paisley *et al.*, 1978) or variable depending if the palpation was performed before or after 40 days of pregnancy (Vaillancourt *et al.*, 1979). This is important because the embryo/fetus can be affected

by factors other than palpation per rectum. The progesterone level is high in conditions other than pregnancy, as presence of luteal cysts, long estrous cycles, sampling during luteal phase, pyometra (Pennington *et al*., 1976) as well as in a pregnant females with embryo/fetus death (Kassam *et al*., 1987). In addition, progesterone level is a better indicator of "non pregnancy" status than for pregnancy status (Shemesh *et al*., 1978; Laing *et al*., 1980). Bovine pregnancy specific protein B (bPSPB), a glycoprotein produced by the trophoblast, persist elevated despite embryo/fetus death or embryos in the process of degeneration (Maurer *et al*., 1985; Humblot *et al*., 1988b). In spontaneous or induced embryo/fetal mortality elevated levels of progesterone or protein B (Kassam *et al*., 1987; Maurer *et al*., 1985) as well as positive signs of pregnancy persisted for several days despite the embryo/fetal death (Parmigiani *et al*., 1978; Kassam *et al*., 1987). Differences among farms are well established and are more related to management factors than to infectious diseases (Thompson *et al*., 1994). This was not taken into consideration in some studies. Most of the previous reports pool together heifers with cows. One study showed that pregnant heifers have lower embryo/fetal mortality rates than cows (Labernia *et al*., 1996) however, these data were retrospective and with no control group. Previous studies did not report the number of twin pregnancies. Twin pregnancies increase the risk of embryo/fetal death and abortion (Day *et al*., 1995). Moreover, in previous studies, real practice conditions were not followed. For example the females were palpated per rectum by more than one person at the same time, different techniques were used at the same time, or different techniques were used in the same animal by more than one person (Abbitt *et al*.., 1978; Paisley *et al*.., 1978; Vaillancourt *et al*.., 1979; Franco *et al*.., 1987). In the above cases, the procedure of palpation per rectum was more invasive than the one used for diagnosis of pregnancy in practice. In summary, all the previous studies have several confounding factors: viability of embryo/fetus was unknown, absence of a control group (non-palpated per rectum group), interval to evaluate the palpation per rectum, differences among farms, differences among categories (cows versus heifers), presence of twins, procedure of palpation per rectum and number of sick/ill females.

Confirmation of pregnancy status before or at the time of palpation per rectum by another method allows the differentiation of naturally occurring embryonic/fetal loss from embryonic/fetal loss potentially induced by palpation per rectum. The use of transrectal ultrasonography permits an earlier pregnancy diagnosis than palpation per rectum, gives immediate information about the presence of positive signs of pregnancy as well as on embryo/fetus viability and reduces the number of false positive diagnoses and false negatives when palpation per rectum is used (Romano and Magee, 2001).

SPONTANEOUS EMBRYO/FETUS MORTALITY

In 1914, Hammond recognized that embryo/fetus loss was common in livestock. After 90 years the basic causes of embryo/fetus death continue to be poorly understood. Embryonic/fetal mortality is the most important factor that reduces the reproductive efficiency in cattle (Ayalon, 1978; Wilmut and Sales, 1981; Peters, 1996). The importance of this loss is not only technical but also economical (Thurmond and Picanso, 1993). Data from old reports had estimated the cost of this pregnancy loss at around $ 1.4 billion in the USA (Gerritts *et al.*., 1976) and £ 250 million in the UK per year (Peters and Ball, 1995). It has been estimated that the cost of an abortion amounts to $ 640 (Thurmond *et al.*., 1990). The cost to a dairy with 600 pregnancies per year could be as high as $ 31,200 per year (Thurmond and Picanso, 1990). In dairy cows the average calving rate for each insemination is approximately 50%, and in large commercial dairy operations in the US frequently close to 30-40% (MacMillan *et al.*., 1996; Lucy, 2001). The fertility rate in dairy cattle has declined compared to 20-25 years ago (Lucy, 2001) therefore each pregnancy is now even more valuable. In cattle, the embryonic period is relatively short (until day 45; Committee on Bovine Reproductive Nomenclature, 1972) and most of the losses occur during this period. (Ayalon, 1978; Kummerfeld *et al.*., 1978; Diskin and Sreenan, 1980; Maurer and Chenault, 1983; Humblot, 1986). Fertilization failure is included in the above percentage. On average, in normal healthy cattle, fertilization rate is approximately 85 to 90% (Boyd *et al.*., 1969;

Diskin and Sreenan, 1980). Therefore, fertilization failure is not a very significant percentage of the overall pregnancy loss. However, new information originated from dairy cattle indicates that fertilization rate is reduced in lactating dairy cattle (Sartori *et al.*, 2002). Most of the embryos are lost well before the critical time of pregnancy recognition (day 16), therefore, the estrous cycle length is not affected (Maurer and Chenault, 1983; Humblot and Dalla Porta, 1984). The major proportion of this embryo loss seems to occur between days 8 and 16 (Diskin and Sreenan, 1980). In general, embryonic mortality is suspected when the interval between insemination and return to estrus exceeds the normal 18 to 24 day range (Kummerfeld *et al.*, 1978) or when the levels of progesterone either in milk or blood progesterone show a decline after day 25 (Humblot *et al.*, 1983). This interval is affected by the quantity and quality of the estrus detection method used. Most of the information published deals with infectious, environmental factors and iatrogenic factors of embryo/fetal loss (Parmigiani *et al.*, 1978; Woelffer, 1981; Drost and Thatcher, 1994; Vanroose *et al.*, 2000). The cause of pregnancy loss is generally unknown in most cases. In the USA, from 3,812 abortions only 23.3% had a determined etiology (Hubbert *et al.*, 1973). Another study showed that from 2,544 abortions 35.3% had a determined etiology (Kirkbride *et al.*, 1973). In Australia, from 265 abortions only 37% had a defined cause (Jerrett *et al.*, 1984) while in Canada, in only 23% of 227 abortions was a cause detected (Mitchell, 1960). In England, also a high proportion of the cases remained unknown (Johnson, 1983). In summary, all these studies agree that in the majority of the cases of abortion the diagnosis of its etiology is undetermined (perhaps around 30% in various laboratories) and that if determined infection is one of the most frequent causes (Miller, 1986). Nevertheless, the presence of a detected microorganism does not necessarily mean that it is the cause of the abortion. Moreover, most of the reports referred to a second or third trimester gestation, in which the abortions (fetus, placenta) are easily observed. Moreover, many of the studies refer to the epidemic type of abortion, that is, the one caused by infectious agents and the ones that correspond to a mid to term fetus (late pregnancies) in which the abortion is easily observed. However, the majority of the

pregnancy losses (the endemic type) occur during early gestation (Ayalon, 1978; Peters, 1996) when the abortions go unobserved; when the only signs of pregnancy loss are a return to estrus or in a further examination in which the female is found open or does not calve at the expected time. As stated above, many of the studies that tried to determine the etiology of pregnancy loss have done so by studying mid to term fetus losses. These studies reported an identifiable cause in approximately 20 to 37 %. Nevertheless if we take into account all pregnancy losses, including embryo and early term fetuses, the identifiable causes of abortion would be much lower.

In modern dairy practice lactating cows continue to experience a high level of pregnancy loss, despite intensive efforts towards reducing specific infectious diseases through preventive heath programs that include strict protocols of immunization against specific pathogenic agents and other sanitary measures. The endemic nature of this loss suggests that factors other than or in addition to pathogens may contribute to the risk of abortion (Thurmond *et al*., 1990; Forar *et al.*, 1995). Little is known about late spontaneous embryo/fetal mortality in cattle and few studies provide a description of the process (Kastelic *et al*., 1991; Forar *et al.*, 1995). Moreover, in *in vitro* produced bovine embryos, especially in cloned embryos, embryo/fetal mortality during the first trimester of pregnancy is much higher than in *in vivo* produced pregnancies. This high embryo/fetal mortality is currently reducing the efficiency of this new reproductive biotechnology (Hill *et al*., 2000).

Factors that were previously found to influence embryo/fetus mortality are chromosomal and genetic defects (Mylrea, 1963; Bishop, 1964; King, 1990; Kawarsky *et al*., 1996), nutrition (Dunne *et al.*, 1999; Laven and Drew, 1999), maternal age (Erb and Holtz, 1958; Ball, 1978), environmental factors (Wise *et al*., 1988; Ryan *et al.*, 1993), male effect (Hawk *et al.*., 1955a; Bishop, 1964), time of insemination (Pursley *et al*., 1998), hormonal imbalance (Ayalon, 1978; Lafrance *et al*., 1989), infectious diseases (Drost and Thatcher, 1994), uterine environment (Ayalon, 1978; Wiebold, 1988), iatrogenic (palpation per rectum: Abbitt *et al*., 1978; insemination of pregnant cows: Weaver *et al*., 1989; prostaglandin F-2α administration: Cavestany and Foote,

1985). Numerous attempts have been made to prevent embryo/fetal mortality in ruminants with exogenous hormones, including progesterone (Wiltbank *et al*., 1956; Robinson *et al*., 1989), interferons (Nephew *et al*., 1990), gonadotrophin releasing hormone (GnRH; MacMillan *et al*., 1986) and human chorionic gonadotrophin (hCG; Breuel *et al*., 1989; Nishigai *et al*., 2002), all of which have produced variable results (Jubb *et al*., 1990; Lewis *et al*., 1990; Van Cleeff *et al*., 1991; Humblot *et al*., 1993).

Despite many years of study, the causes of reproductive failure in farm animals are poorly understood. Most investigations have underestimated the incidence of pregnancy loss, but few investigators have attempted to identify when during gestation the conceptus dies and the causes of death. Searching for answers, the pathologist has looked for infection; the endocrinologist for hormonal imbalances, the geneticist for chromosomal aberrations, and so on through the various associated disciplines. Each search has usually yielded some suggestion of a possible physiological or pathological cause of embryo/fetal death without any definitive answer. Until a greater understanding of embryo/fetal mortality is reached, little can be done to improve the reproductive efficiency, design preventive measures and treatments and prevent economic loss.

CLINICAL , BEHAVIORAL AND ULTRASONOGRAPHIC CHARACTERISTICS OF FEMALES WITH EMBRYO/FETUS MORTALITY

Embryonic/fetal mortality, as mentioned, has a major impact on reproductive efficiency in cattle (Ayalon, 1978). Most of the information published deals with infectious, environmental and iatrogenic factors of embryo/fetal loss (Drost and Thatcher, 1994; Vanroose *et al*., 2000) but non-infectious causes probably account for more than 70% of the embryo/fetal death (Vanroose *et al*., 2000). Spontaneous embryo/fetal mortality is defined as that which occurrs in an apparently healthy cow/heifer and that is not related to a specific cause. Spontaneous embryo/fetal mortality is the most important component in the reduction of reproductive efficiency. Little is known about late spontaneous embryo/fetal mortality in cattle. Based in clinical

observations in cattle practice there seems to are two types of spontaneous embryo/fetal mortality based on palpation per rectum and ultrasonographic images of the embryo/fetus (Romano, unpublished observations). These two types of embryo/fetal mortality were named Type I and Type II. Type I was first noted and was characterized by the presence of the allantochorion membrane by palpation per rectum (positive fetal membrane slip), presence of embryo/fetus degeneration and a functional corpus luteum. Type II was characterized by absence of an embryo/fetus, absence of positive fetal membrane slip and the presence or absence of a functional corpus luteum. Embryo/fetal mortality Type I was treated with prostaglandin F-2α to induce luteolysis in order to clean the uterus. Females with embryo/fetal mortality Type II were either treated with prostaglandin F-2α if a functional CL was observed or allowed to breed as soon as they showed standing estrus. Therefore, in this preliminary study, no follow-up at that time on the clinical evolution of each of the types of embryo/fetal mortality was done. This knowledge could help to understand these types of embryo/fetal mortality, evaluate the reproductive and productive implications and design future preventive measures in order to increase reproductive efficiency

EMBRYO/FETUS MORTALITY IN NUCLEAR TRANSFER DERIVED EMBRYOS

Nuclear transfer (NT) is a technique that allows the production of embryos, fetuses, and offspring from embryonic, fetal, and adult derived cell types in a variety of species (Wilmut *et al*., 1997; Baguisi *et al*., 1999; Polejaeva *et al*., 2000; Lin *et al*., 2001; Wakayama *et al*., 1998; Westhusin *et al*., 2003). Success in the production of viable offspring by nuclear transfer with somatic cells has been achieved in cattle (Kato *et al*., 1998), sheep (Wilmut *et al*., 1997), goats (Baguisi *et al*., 1999), pigs (Polejaeva *et al*., 2000), horses (Galli *et al*., 2003), mules (Woods *et al*., 2003), mice (Wakayama *et al*., 1998), rats (Roh *et al*., 2003), rabbits (Chesne *et al*., 2002), cats (Shin *et al*., 2002)

and in wild and endangered species: gaur (Vogel, 2001), mouflon (Loi *et al*., 2001) and white tailed deer (Westhusin and Kraemer, unpublished observation).

The production of cloned embryos by nuclear transfer has many agricultural, conservational, pharmaceutical, and therapeutic applications (Wolfe and Kraemer, 1992; Lewis *et al*., 1998; Colman and Kind, 2000; Westhusin *et al*., 2003). NT in which the oocyte nucleus is replaced with the nucleus of a developmentally more advanced cell is being developed as a powerful tool for resolving fundamental biological questions of development and differentiation (Campbell *et al*., 2001). The use of transgenic cell lines will permit the production of animals that express specific substances through their milk i.e. coagulation factor IX and cell lines that do not express histocompatibility antigens. This will allow the production of recombinant factor IX (its deficiency results in Hemophilia B) in milk which will provide an alternative source of this substance at a lower cost and free of the potential infectious risk associated with products derived from human blood (Schnieke *et al*., 1997). In addition, transpecie tissue and organ transplantation (xenotransplantation) could become a more successful technique (Colman and Kind, 2000). Moreover, the use of nuclear transfer derived embryos from animals (male or females) in which their progeny was already evaluated will permit the wide use of these animals (McClintock, 1998). All these applications clearly have great benefits.

The process of embryo reconstruction and production of viable offspring by NT is a multi-step procedure, each of which needs to be performed correctly for the success of the next step. The procedure is of multifactorial design: the final result is the combined action of each step and not the average of all these steps. The ultimate goal of cloning is to obtain healthy fertile offspring (Yanagimachi, 2002). Therefore, the nuclear transfer efficiency should be assessed taking into account the number of live offspring obtained in relation to the total number of blastocyts transferred or to the total couplets (ooplast+donor cells) produced (Yanagimachi, 2002). The former gives a much higher rate than the latter. Some investigators do not report the total number of oocytes that received nuclear transfer, which makes comparisons among studies difficult. The

technique has problems at the different stages: not all the oocytes mature, the enucleation protocol can destroy the ooplast, not all oocytes fuse with the donor cell, not all the couplets are activated and cleave, not all embryos produce blastocysts of transferable quality. The overall efficiency of the technique, however, remains low because only a very limited percentage (0.5-5%) of the transferable embryos result in full-term development (Campbell *et al*., 2001; Yanagimachi, 2002; Oback and Wells, 2003). The inefficiency of the procedures with high rates of embryo and fetal losses and high costs hamper its widespread use in livestock (McClintock, 1998).

In general, early pregnancy loss from *in vivo* produced embryos results from defects of the embryo/fetus, the placenta, alterations of the uterine environment, or the conceptus-maternal interactions (Wilmut and Sales, 1981). Little is known about the causes of the early pregnancy loss in *in vitro* produced embryos. More research in this area will permit the understanding of how to improve results with this new reproductive technique.

A high frequency of post-implantation developmental arrest after the transfer of morphologically normal blastocysts has been detected in embryos produced by nuclear transfer (Stice *et al*., 1996; Hill *et al*., 2000; Campbell *et al*., 2001; Yanagimachi, 2002). In many of the pregnancies carrying these embryos, the embryo/fetal losses were associated with placental abnormalities during the first trimester of pregnancy (Stice *et al*., 1996; Hill *et al*., 2000). One of the causes of abnormal placentation could be reduced number of trophectoderm cells, which originated the placenta and other extraembryonic membranes, as suggested by Koo *et al*., (2002). In addition, nuclear transfer derived embryos have a marked increase in chromosome abnormalities compared with *in vivo* or *in vitro* produced embryos (Slimane and King, 2002). The number of chromosomal abnormalities was higher in nuclear transfer derived embryos compared with the donor cells that are used to produce them (Slimane *et al*., 2003). Other causes might be abnormal gene expression as detected in cow placenta cells (Hashizume *et al*., 2002). In a recent study, Hill *et al*., (2002) reported the expression of major histocompatibility complex (MHC) class I in placenta cells during early pregnancy. These antigens are not

expressed except during late pregnancy in normal conditions (Low *et al*., 1990; Davies et al., 2000). In addition, the *in vitro* culture medium in which the nuclear transfer derived embryo is maintained until the morula/blastocyst stage has been shown to be a cause of abnormal development in non-nuclear transfer *in vitro* produced embryos (Thompson, 2000). In summary, the abnormal placentogenesis can be due to the multiple and combined action of the different factors mentioned above.

Calves produced from nuclear transfer embryos have been characterized by high birth weight and low survival rate (Bondioli *et al*., 1990; Keefer *et al*., 1994). The cause of these problems has not been identified. Calf size at birth varies within clutches of genetically identical embryos, and the incidence of abnormally large calves approaches 20-30% of the calves born (Bondioli *et al*., 1990; Wilson *et al*., 1995). Studies in mammals suggest that fetuses with abnormal intrauterine growth resulting either in abnormally large or small newborns, develop more perinatal complications and have greater difficulties in adjusting to extra-uterine life in the immediate postpartum period (Holland and Odde, 1992). Calves produced by nuclear transfer had a birth weight that was 20% higher than *in vivo* produced embryos (either produced by embryo transfer or artificial inseminsation). The variability in birth weight for nuclear transfer embryos was four to twelve folds greater than calves produced *in vivo*. Nevertheless this accelerated growth did not continue beyond birth (Wilson *et al*., 1995). Most of the information available about large sized NT conceptuses came from late gestation, calving and the neonatal period (Garry *et al*., 1996; Bondioli *et al*., 1990; Wilson *et al*., 1995).

Hormonal environment seems to affect the conceptus growth. An accelerated intrauterine growth of bovine embryos was obtained when a cow received exogenous progesterone for four days, starting at 36 hours following mating. The accelerated embryonic growth was evident 14 days post-breeding (Garrett *et al*., 1988). In sheep, an accelerated embryonic development was obtained when embryos were transferred into an intermediate host uterus which was chronologically 3 days advanced (Wilmut and Sales, 1981). Closer study of nuclear transfer derived pregnancies during the early period of pregnancy might be able to detect abnormal growth in these conceptuses.

Previous studies in embryos/fetuses produced by artificial insemination suggest the presence of two types of embryo/fetus death in cattle (Romano, unpublished observations). One was characterized by the presence of a functional corpus luteum, positive signs of pregnancy by palpation per rectum and signs of embryo/fetus degeneration by transrectal ultrasonography (named: embryo/fetus mortality Type I). The other was characterized by the absence of a functional corpus luteum, negative signs of pregnancy by palpation per rectum and absence of signs of embryo/fetus degeneration by transrectal ultrasonography (name: embryo/fetus mortality Type II). In the first case, the placenta seemed to continue to send information to the endometrium that a conceptus is there, even though the embryo/fetus was detected as dead approximately three weeks earlier. On the contrary, in the second case, the uterus does not recognize the presence of a live embryo/fetus inside, because in general in the last evaluation the heart beat, movement of the fetus or umbilical vessel pulsation was observed in the embryo/fetus. Therefore, if the cause of embryo/fetus death is due to an earlier triggering in the luteolytic mechanism from the uterus the exogenous administration of progesterone or progestagens might be a potential effective treatment to maintain the progestational characteristics of the uterus necessary to rescue these valuable embryos.

Protein B is a glycoprotein hormone produced by the giant cells of the trophoblast that appears in blood after day 15 post-breeding and consistently at days 24 to 28 after breeding (Sasser *et al.*, 1986). Due to that fact, this specific protein of pregnancy produced by the trophoblast giant cells it is a useful marker of a viable placenta. Progesterone is a hormone produced by the corpus luteum and is involved in the establishment and maintenance of the uterine characteristics necessary for pregnancy

(McDonald *et al*., 1953). The use of luteolytic drugs during the first 5 months of gestation will cause regression of the corpus luteum, resulting in abortion between 2 and 7 days (Barth, 1986; Kastelic and Ginther, 1989). The use of progesterone or progestagens has been effective in maintaining pregnancy after luteolysis, enucleation of corpus luteum or bilateral ovariectomy (Tanabe, 1966; Zimbelman and Smith, 1966; Lulai *et al*., 1994). The use of protein B as a hormonal marker of placental hormonal function during early pregnancy could help us understand how to monitor placental abnormalities in nuclear transfer pregnancies. In addition, progesterone levels, may provide information about the role of the corpus luteum during the pregnancy loss found in these clone pregnancies.

RESEARCH PROJECT RATIONALE

The broad objective of this project was to improve our knowledge about early pregnancy diagnosis, effect of palpation per rectum on embryo/fetus viability and factors that influence the risk of spontaneous embryo/fetus mortality of *in vivo* and nuclear transfer derived embryos. This project was conducted in five experiments, each with a specific objective.

The first experiment, evaluated the accuracy of transrectal ultrasonography for early pregnancy diagnosis in cattle. The second experiment, estimated the effect of palpation per rectum on embryo/fetus viability. The third experiment, estimated the frequency and factors (i.e. maternal age, lactation number, single/twin pregnancies) that affect the risk of spontaneous embryo/fetus death. The fourth experiment, describes the clinical and ultrasonographic findings of the process of embryo/fetus death. Finally, the fifth experiment estimated the frequency, growing rate and type of embryo/fetus death in nuclear transfer embryos.

CHAPTER II

EARLY PREGNANCY DIAGNOSIS BY TRANSRECTAL ULTRASONOGRAPHY IN DAIRY CATTLE

INTRODUCTION

An important step in dairy management is to examine for pregnancy within 45 days after breeding (Zemjanis, 1971). Diagnosis of non-pregnancy prior to the second expected estrus will allow making a management decision before the next estrus (Zemjanis, 1971). Pregnancy diagnosis at a later stage insures loss of an additional 18-24 days if the cow is not pregnant. The reduction of open days is one of the major objectives in the dairy cattle industry (BonDurant, 1986). Thus, an accurate method of determining pregnancy/non-pregnancy before the expected second estrus would be ideal (Studer, 1969; Zemjanis, 1971). The main objective of a reproductive health program is to make sure that healthy cows calve at 12-13 month intervals to optimize their lifetime milk production. Seegers and Malher (1996) reported that the daily cost of a cow that persists open past day 100 (after calving) is between $ 2.50 and 4.00 to dairy producers. Early pregnancy diagnosis can assist dairyman in managing open cows and improving reproductive performance and economics of their herd (Oltenacu *et al*., 1990). Early detection of non-pregnancy should lead to earlier intervention and consequently shorter, more economical calving intervals. It was shown that the earlier the pregnancy diagnosis is performed, the more profitable is the return (Oltenacu *et al*., 1990).

In bovine practice, two methods allow us to immediately diagnose pregnant/non-pregnant females: palpation per rectum and transrectal ultrasonography. Palpation per rectum as a direct method for pregnancy diagnosis is performed 30 days after breeding/artificial insemination. However, neither critical studies in regard to its accuracy at earlier stages nor comparing it with transrectal ultrasonography are available. It is considered that a good practitioner is able to detect pregnant/non-pregnant

animals from day 35 on (Zemjanis, 1971; Roberts, 1986; Momont, 1990). Transrectal ultrasonography for pregnancy diagnosis offers some advantages over palpation per rectum: earlier diagnosis of pregnancy/non-pregnancy, determination of embryo/fetus viability, reduction of misdiagnosis (false negatives and false positives) and reduction of "potential" iatrogenic embryo/fetal attrition (Romano and Magee, 2001). As with all diagnostic techniques, there are two main concerns regarding early use of transrectal ultrasonography for pregnancy diagnosis: safety and accuracy. Reports have shown transrectal ultrasonography to be a safe technique that does not affect embryo/fetus viability (Kahn, 1992; Ball and Longue, 1994; Baxter and Ward, 1997). Nevertheless accuracy has not been clearly established. As it was mentioned above, a correct method of pregnancy diagnosis is not only based in the correct "pregnant" but also in the correct "non-pregnant" diagnosis. In order to increase the accuracy of the non-pregnant diagnosis, a procedure with high negative predictive value that eliminates the possibility of false negatives is required. Consequently, a diagnosis of non-pregnancy can be made with confidence and immediate action taken, such as: treatment, synchronization, culling or sale.

The use of protocols for estrous synchronization with artificial insemination at a fixed time (Wiltbank, 1998; Stevenson *et al*. 2000) requires an early and accurate method of non-pregnancy diagnosis in order to enroll the animals in a new round of estrous synchronization. In embryo transfer programs, the accuracy of pregnancy/non-pregnancy diagnosis will enable open recipients to be returned or excluded from the recipient herd as soon as possible. Some studies recommended the use of transrectal ultrasonography at 25 or 26 days after breeding to determine the pregnancy/non-pregnancy status (Fissore *et al*., 1986; Pieterse *et al*., 1990; Fricke, 2002; Lares *et al*., 2002). However, these studies are in contrast with previous reports (Badtram *et al*., 1991; Hanzen and Laurent, 1991) and our experience. At 25-26 days post-insemination the sensitivity and negative predictive values reported are far from being 100 % (Badtram *et al*., 1991; Szenci *et al*., 1995; Filteau and DesCôteaux, 1998), therefore, the possibility of diagnosing an animal incorrectly as non-pregnant (false negative) is highly

probable. These females incorrectly diagnosed as non-pregnant will receive prostaglandin F-2α which will cause immediate abortion, therefore, these animals will never be detected as being pregnant. The induction of iatrogenic abortion for misdiagnosis is not acceptable technically and economically (Thurmond and Picanso, 1990) especially now that fertility in lactating dairy cattle has declined compared to 20-25 years ago (MacMillan *et al*., 1996; Lucy, 2001). The negative predictive value of an early pregnancy diagnosis should therefore be 100% to avoid inducing early abortion, culling or selling "unnecessarily", pregnant females. Few studies have been designed to evaluate the best day of pregnancy diagnosis with the maximum sensitivity and negative predictive value. In our experience, under non-controlled situations, heifers were able to be detected earlier than cows with confidence as pregnant/non-pregnant animals. Differences in uterine characteristics as well as in uterine position are probably the factors that allow an earlier detection in heifers. In most of the studies, cows or heifers were used (Kastelic *et al*., 1989; Willemse and Taverne, 1989; Pieterse *et al*., 1990; Badtram *et al*., 1991; Hanzen and Laurent, 1991; Filteau and DesCôteaux; 1998) or both categories were analyzed together (Chaffaux *et al*., 1986; Hansen and Delsaux, 1987). Few studies comparing cows versus heifers were found (Hughes and Davies, 1989; Badtram *et al*., 1991; Hanzen and Laurent, 1991). In one report, a negative correlation between age of the female and accuracy of pregnancy diagnosis in females evaluated by transrectal ultrasonography at 4 weeks was noticed (Hughes and Davies, 1989), however, in two further studies no differences were found (Badtram *et al*., 1991; Hanzen and Laurent, 1991).

The objectives of the present study were: first, to determine the day of maximum sensitivity and negative predictive value of transrectal utltrasonography (TRUS) for pregnancy and second, to determine if there is a difference between heifers and cows.

MATERIAL AND METHODS

This study was conducted in the Dairy Cattle Center of Texas A & M University. Holstein and Jersey breeds were used. After passing a voluntary waiting period of 55 days (after waiting 55 days postpartum) the cows were included in the estrous synchronization program for artificial insemination. Lactating cows were housed in concrete dry lot covered free stalls, milked twice daily, and fed a total mixed diet consisting of corn or grain sorghum, soybean meal, alfalfa, and corn silage. Mineral salt and water were offered at libitum. Diets were formulated to meet or exceed NRC requirements (1989). Estrus detection was performed twice a day: early in the morning and late in the afternoon. Artificial insemination was performed according to the am/pm rule (estrus= day 0). Cows were randomly divided in order to have a TRUS once between days 24 to 30. Heifers were divided at random in order to have TRUS once between days 21 and 27. A total of one thousand four hundred TRUS in Holstein and Jersey females were performed (1079 in cows and 321 in heifers). Some of the animals detected opened by TRUS were estrous synchronized, inseminated and included again in the study. The number of TRUS performed in cows in first, second, third and fourth and over lactations were 531, 279, 169 and 100, respectively. The cows were restrained in a stanchion in partial dim ambient light while examined. During the procedure the operator removed the feces from the rectum, introduced the probe and scanned the uterine horns and ovaries. The transducer and the sleeved arm of the examiner were lubricated with an obstetrical lube to facilitate penetration through the anal sphincter and to establish good contact with the floor of the rectal mucosa. The transducer was positioned dorsal to the genital tract and slowly advanced cranially. The cervix, right uterine horn, the left uterine horn and the uterine body were examined for signs of pregnancy. The experienced operator tried to scan the same area twice. All ultrasonographic examinations were performed by the same operator. A portable Aloka 500 SSD with a 5 MHZ linear transducer specially adapted for transrectal examination in large domestic animals was used. The probe was cleaned between examinations. A cow

or heifer was considered presumptively as pregnant between days 24 to 26 or days 21 to 23, respectively, when the presence of fluid of varying amounts into the lumen of an echogenic uterine horn plus the presence of a corpus luteum ipsilateral, was seen. The observation of an allantochorion or embryo confirmed the diagnosis of pregnancy. Every transrectal ultrasonography was subsequently compared with another TRUS performed some days later (between 3 and 8 days) but always, the second TRUS, was performed after day 30 for cows and day 27 for heifers. These days were chosen as preliminary observations had shown that the accuracy of the technique for detecting pregnant and non pregnant females was 100% after day 31 for cows and 28 for heifers. The operator was required to make the diagnosis of pregnancy/non-pregnancy without knowing previous results. Sensitivity is the capacity of the test to detect correctly pregnant females from the total pregnant females. Specificity is the capacity of the test to detect correctly the non pregnant females from all the non pregnant ones. Positive predictive value is the capacity of the test to predict correctly the pregnant ones while negative predictive value is the capacity of this test to correctly predict the opened or non pregnant females. A correct diagnosis was defined as: 1-Cow/heifer diagnosed pregnant with TRUS and subsequently confirmed pregnant in the next TRUS (a). 2-Cow/heifer diagnosed non-pregnant with TRUS and subsequently confirmed non-pregnant in the next TRUS (c). An incorrect diagnosis was defined as: 1-A cow/heifer diagnosed non-pregnant with TRUS and subsequently confirmed pregnant in the next TRUS (false negative; d). 2-A cow/heifer diagnosed pregnant with TRUS and subsequently confirmed non-pregnant in the next TRUS (false positive; b). From these, the sensitivity (a/a+d) X 100, specificity (c/c+b) X 100.; positive predictive value (a/a+b) X 100; and negative predictive value (c/c+d) X 100 of the TRUS pregnancy test were calculated.

The statistical analysis used for comparisons among days for each measurement was analyzed using proportions by Chi-square or Fisher exact test for 2 by 2 tables with at least one cell having fewer than 5 observations. A difference was considered statistically significant at $P<0.05$ (Devore and Peck, 1993).

RESULTS

The summary of results for cows is shown in Table 1 and for heifers in Table 2. In cows, the sensitivity increased gradually from 74.5% at day 24 to 100 % at day 29 ($P<0.01$). At the same interval the specificity increased from days 24 and 25 and then was maintained around 96.6% (range from 95.6 to 97.4%)($P<0.01$). The positive predictive value increased in the first two days of evaluation and after day 26 was stable (mean from days 26 to 30 = 91.5 %; range from 89.1 to 93.6%)($P<0.01$). The negative predictive value increased from 88% at day 24 to 100% at day 29 ($P<0.001$). In heifers, the sensitivity increased from 50% at day 21 to 100% at day 26 ($P<0.01$). The specificity increased from 87.5% at day 21 and from days 23 on, was maintained around 94%

Table 1.Comparison among the accuracies of transrectal ultrasonography performed using a 5-MHz linear transducer in dairy cows.

Days of TRUS	Day 24	Day 25	Day 26	Day 27	Day 28	Day 29	Day 30	Total
Number of TRUS[1]	150	155	162	153	157	151	151	1079
Pregnant correct	35	57	44	45	41	42	34	298
Pregnant Incorrect	10	12	3	4	5	4	3	41
Not pregnant correct	93	80	114	101	110	105	114	717
Not pregnant incorrect	2	6	1	3	1	0	0	21
Sensitivity*	74.5	90.5	97.8	93.8	97.6	100	100	92.8
Specificity*	90.3	86.9	97.4	96.2	95.6	96.3	97.4	94.6
PPV*	77.8	82.6	93.6	91.8	89.1	91.3	91.9	87.9
NPV*	88.6	93.0	99.1	97.1	99.1	100	100	96.7

* values are shown in %

(range from 87.9 to 96.5%)(P>0.05). The positive predictive value (PPV) was increased after the first two days of evaluation and from day 23 on was 92.8% (range from 86.7 to 96.7%)(P>0.05). The negative predictive value (NPV) of 63.6% at day 21 increased to 100% at day 26 (P<0.01). The maximum sensitivity and negative predictive value was obtained three days earlier in heifers (day 26) than in cows (day 29).

DISCUSSION

Few studies address sensitivity, specificity, positive and negative predictive values of TRUS in cattle for pregnancy diagnosis. Some reports suggest the possibility of confirming pregnancies using transrectal ultrasonography between days 9 and 21 (Curran *et al*., 1986a and 1986b; Boyd *et al*., 1988; Totey *et al*., 1991); however, neither sensitivity nor specificity values were reported among days. Moreover, some of these

Table 2. Comparison among the accuracies of transrectal ultrasonography performed using a 5-MHz linear transducer in dairy heifers.

Days of TRUS	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27	Total
Number of TRUS[1]	16	41	50	53	60	48	53	321
Pregnant correct	4	12	18	22	26	17	25	124
Pregnant incorrect	1	3	1	1	4	1	2	13
Not pregnant correct	7	22	26	28	29	30	26	168
Not pregnant incorrect	4	4	5	2	1	0	0	16
Sensitivity*	50.0	75.0	78.3	91.6	96.3	100.0	100.0	88.6
Specificity*	87.5	88.0	96.3	96.5	87.9	96.7	92.8	92.8
PPV*	80.0	80.0	94.7	95.6	86.7	94.4	92.6	90.5
NPV*	63.6	84.6	83.8	93.3	96.6	100.0	100.0	91.3

*values are shown in %

studies were performed under optimal research conditions, using heifers, ovulation time as day 0 and often with repeated examinations. However, these criteria are almost impossible to satisfy under practical conditions. Other studies recommend the use of TRUS at day 25 or 26 to determine pregnancy/non-pregnancy (Fissore *et al*., 1986; Pieterse *et al*., 1990; Fricke, 2002; Lares *et al*., 2002). However, some of these studies used few animals, only one category of animal and did not critically test the sensitivity, specificity and positive and negative predictive values. The present study was specifically designed to address these issues. Therefore in our study: 1) A sufficient number of transrectal ultrasound procedures were performed; 2) cows and heifers were analyzed separately; 3) the results were analyzed on a day to day basis; 4) 5 MHz linear transducer was used by a trained person; 5) the second diagnosis was by another transrectal ultrasonography; 6) the interval between diagnosis was short, between 3 and 8 days and always after day 30 for cows and day 27 for heifers. The rationale for all this approach was: using a sufficient number of females to permit a sound evaluation. Cows were analyzed separately from heifers for two reasons: the position of the genital tract changes with the reproductive age (Zemajnis, 1971) and the embryo/fetal mortality appeared to be different between the two categories (Chapter III and IV). The results were analyzed daily in order to clearly estimate the sensitivity, specificity, negative and positive predictive values of TRUS per each day of the study. Transrectal ultrasonography was used in the second diagnosis because it is a safe and accurate method that permits earlier pregnancy diagnosis than palpation per rectum (Romano and Magee, 2001). Palpation per rectum was not used because information about its accuracy during early stages of pregnancy is not available and there is the potential deleterious effect on embryo viability as suggested by some studies (Abbitt *et al*., 1978; Paisley *et al*., 1978; Vaillancourt *et al*., 1979; Franco *et al*., 1987). A 5 MHz linear transducer was used because it is the most frequently used transducer in practice and in previous reports (Willemse and Taverne, 1989; Pieterse *et al*., 1990; Badtram *et al*., 1991; Hanzen and Laurent, 1991; Szenci *et al*., 1996; Filteaux and DesCoteaux, 1998). The interval between first and second diagnosis was reduced in order to avoid other confounding

factors such as embryo mortality. This could affect the percentages of false positives especially if long intervals are used. Spontaneous embryo mortality is highest during the first 45 days of pregnancy (Chapter III and IV). Therefore, the longer the interval between first and second diagnosis the higher will be the probability of having a high number of false positives due to embryo mortality. Consequently, a reduction in specificity and positive predictive values would occur.

In the present study, the sensitivity of transrectal ultrasonography increased gradually form days 24 to 30 in cows and from days 21 to 27 in heifers. The maximum sensitivity was reached at day 29 in cows and day 26 in heifers due to the improvement in the ability to detect correctly all the pregnant females. No false negatives were noted when 219 cows were diagnosed as open at days 29 and 30. At day 28 only one cow was misdiagnosed as non-pregnant yielding a 0.3% false negative rate from days 28 to 30 (1/330). In heifers, only one false negative occurred at day 25, and none at days 26 and 27 (1.16%; 1/86). These results are in agreement with our previous experience that no false negative results were obtained starting at day 31 for cows and from day 28 for heifers (JE Romano, unpublished observations). Analysis of the records from the cows that produced the false negatives showed that the cow at day 26 (n=1) was in the 3rd lactation, the three from day 27 (n=3) were 1st lactation (with long reproductive tract), 3rd lactation and 4th lactation and the one from day 28 (n=1) was in 3rd lactation. Therefore, 80% of the misdiagnoses of non-pregnancy between days 26 and 28 were animals in the 3rd or more lactation. This confirmed our suspicion that differences between heifers and cows exist. Hughes & Davies (1989), using a 3.5 MHz linear transducer reported that the accuracy of pregnancy diagnosis was inversely proportional to the age of the cow. At four weeks, 100% accuracy was obtained in heifers versus 94.1%, 78.5%, 53.8% and 50% for cows 3, 4, 5, and 6 year-old, respectively. Scenzi *et al*. (1995) found that most of the false-negative diagnoses of pregnancy by TRUS were performed in cows with the uterus positioned far cranially to the pelvic inlet, however, the incorrect negative diagnoses were not influenced by age. Our results are in contrast with previous reports of no differences between cows and heifers using a 5 MHz

transducer (Badtram *et al*., 1991; Hanzen and Laurent, 1991). The reproductive age of the cow was a factor that affected the number of false negatives because the uterus changes in relation to the pelvic inlet with the number of calvings (Zemjanis, 1971). The position and the configuration of uterine horns in older cows creates difficulties in obtaining a clear cross section view of the horn necessary to detect the small amount of fluid that may be present during this stage of pregnancy. In cows from the 2nd lactation onward, retraction of both uterine horns was necessary in some cases to permit a correct and thorough scanning. This additional manipulation is in contrast to a previous report (Kastelic *et al*., 1989) that stated that no retraction of the uterus was required, probably, because heifers were used. From the present study, two components affect the overall sensitivity and negative predictive values and need to be taken into consideration when comparing studies: first, the proportion of heifers/cows and second, the percentage of cows in their third or more lactation. As mentioned above, 80% if the misdiagnoses of non-pregnancy were performed in cows from third or more lactations and this group represented 25% of all transrectal ultrasonography performed in the present study.

Calculated data from 4 reports using 3-3.5 MHz transducers showed that the sensitivity and negative predictive value improved when the interval from AI to pregnancy check was longer. However, the proportion of false negatives was 8.9% at 40-49 days after AI (Hansen and Delsaux, 1987, Chaffeux *et al*., 1986; Taverne *et al*., 1985; Humblot and Thibier, 1984). The analysis of five reports using a 5 MHz transducer from days 23 to 33 (Willemse and Taverne, 1989; Pieterse *et al*., 1990; Hanzen and Laurent, 1991; Szenci *et al*., 1996; Filteaux and DesCoteaux, 1998) showed that the sensitivity was 91.5 ± 11.6 (mean ± SD; range 68.8 -100%) and that the negative predictive value was 86.9 ± 12.2 (range: 71.7 – 100%). Differences among reports vary widely due to different conditions in which the ultrasound was performed, breed and experience of the operator working with the equipment. Despite these controversies all studies agree that the sensitivity and negative predictive value increase gradually. Only one report presented the results on a daily basis from days 22 to 40 after AI (Filteaux and DesCoteaux, 1998). In the other reports data were presented in 3 to 10 day intervals.

Therefore, it was not possible to critically compare those results with the present study. In Hanzen & Laurent (1991) 22% of false negatives were found before day 30, however, the number of false negatives decreased to 15.9%, 5.8% and 4.7% for the intervals 30-39, 40-49 and 50-59, respectively. In the Filteaux and DesCoteaux report (1998), using cows only, 100% sensitivity and negative predictive value was reached consistently after day 32. In Pieterse *et al*. (1990) 97.7% sensitivity (n=43/44) was obtained between days 26 to 33, however, there is no mention of the day in which the false negative occurred. In Willemse and Taverne (1989), 100% sensitivity was obtained at day 26-29, however, at days 30-33 the sensitivity dropped to 94.7 and the negative predictive value to 94.1%. Kastelic *et al*., (1989) using only heifers under optimal conditions and using ovulation time as day 0 reached 100% of sensitivity at day 20-22. In the present study, the maximum sensitivity and negative predictive value was reached at days 26 in heifers and 29 in cows. The calculated data from 2 reports using a 7.5 MHz transrectal ultrasonography showed that the sensitivity and negative predictive value improved when the interval from AI to pregnancy diagnosis is longer (Scenzi *et al*., 1995 and 1999). From interval 26 to 32 the values were 85.4% and 86.8 % and from days 33 to 38 the values were 97.9% % and 98.1%, respectively. From the same interval the percentages of false negative were 13.2% and almost 2%, respectively. It is difficult to interpret, however, how when using a high resolution transducer, it took more time to avoid false negatives and also how the number of false negatives at any given date was higher than when a 5 MHz probe was used (Willemse and Taverne 1989; Pieterse *et al*., 1990; Fileteaux and DesCôteaux, 1998) .

From the present study, transrectal ultrasonography showed to be a practical method for early, immediate and accurate pregnancy/non pregnancy diagnosis before day 30 for cows and heifers. This procedure allows for earlier pregnancy diagnosis compared to determination of pregnancy specific proteins. The use of Protein B for testing pregnancy showed 100% of sensitivity and negative predictive value only at days 37/38 (Szenci *et al*., 1998) or later (Humblot *et al*., 1988a). On the other hand, when another hormone secreted by giant cells of the trophoblast, pregnancy associated

glycoprotein 1 ($bPAG_1$) was used, the same level of accuracy was obtained only at days 44/45 (Szenci *et al*., 1998). In addition, transrectal ultrasonography permitted determination of the viability status of the embryo, for which these hormones did not give an immediate indication.

The specificity improved after the first two days of evaluation both in cows (days 24 and 25) and heifers (days 21 and 22). This was probably due to an increase in the amount of allantoic fluid after this stage making it easier to differentiate pregnant from non-pregnant females. The positive predictive value also improved during the same interval for cows and for heifers. The present results are in agreement with Pieterse *et al*. (1990) who found that specificity was different during days 21-25 (82.3%) compared to days 26-33 (87.8). False positive results can be due to two components: misdiagnosis and embryo mortality. The low specificity values encountered in the first 2 days of our study probably include animals in proestrus erroneously diagnosed as pregnant. In some cases, the presence of fluid in the uterus, a regressing corpus luteum and a preovulatory follicle were seen. This was a clear indication of proestrus, and therefore, these were correctly included in the non-pregnant group. However, due to the fact that we did not measure each corpus luteum, the possibility of inclusion of animals around estrus can not be ruled-out. Embryo mortality may be high during these days. In addition, there is the possibility that a diagnoses of pregnancy was done correctly but the embryo was dead or died later, confusing the results. The persistence of false positives during early stages of pregnancy can explain why specificity and positive predictive values never reached 100%. At the present time, there is not a non invasive standard method available for comparison of results simultaneously with transrectal ultrasonography. The present study compared it to transrectal ultrasonography at a later date while others used palpation per rectum or calving records (Taverne and Willemse, 1989; Pieterse *et al*., 1990; Badtram *et al*., 1991; Hanzen and Laurent, 1991; Szenci *et al*., 1995; Filteau and DesCôteaux, 1998). The studies that compared TRUS with palpation per rectum or even calving records have a greater possibility of detecting false positives (due to embryo mortality) which would affect the specificity and predictive values of early TRUS.

Embryo mortality, which is known to be high during the first 45 days of pregnancy, will affect the proportion of false positives using this experimental approach. The prevalence of embryo/fetal mortality is variable among farms (Thurmond and Picanso, 1993; Thompson *et al*., 1994). Embryo mortality was evidently the cause of false positives in later stages of the period of our study. As can be seen from tables 1 and 2, the sensitivity increased during all phases of the investigation period both for cows and heifers. However, the number of false positives persisted. These false positives were probably not due to diagnoses of non-pregnant animals as pregnant but due to correct diagnosis of pregnancies that were later affected by embryo mortality. This was reinforced because in animals diagnosed as pregnant from day 27 on in cows or 25 on in heifers, a viable embryo was always detected.

The negative predictive value improved throughout the investigation period for cows and heifers being highest for cows at day 29 and for heifers at day 26. The positive predictive value increased after the first two days of scanning for both categories and was maintained around the same level during all of the study period. The positive predictive value was always lower than the negative predictive value in cows and from day 25 in heifers. The cause of this difference was probably due to an increase in the accuracy of correct detection of non-pregnant females and to the occurrence of high levels of embryo mortality that produced false positive results. It is necessary to mention, that the predictive values are not only dependent on sensitivity and specificity but also the prevalence of the condition being tested. In the present study, only one herd was evaluated, and the prevalence varies among herds (Thursmond and Picanso, 1993; Thompson *et al*., 1994).

From the results of the present study, as well as from previous reports, differences between sensitivity and negative predictive values on pregnancy diagnosis by TRUS were detected. It is necessary to distinguish between the consistent observation of a pregnancy/non pregnancy (the discriminatory level) from its early detection (the threshold level). Therefore, we recommend that each person must determine their own

threshold and discriminatory level, on the basis of their experience, animal category, equipment resolution and audit of their results.

CHAPTER III

EFFECT OF EARLY PREGNANCY DIAGNOSIS BY PALPATION PER RECTUM ON EMBRYO/FETUS MORTALITY IN DAIRY CATTLE

INTRODUCTION

Early pregnancy diagnosis is vital for a diary management. It should ideally correctly identify pregnant and non pregnant cows as well. Protocols for estrous synchronization with artificial insemination at fixed time (Wiltbank, 1998; Stevenson *et al*. 2000) require an early and accurate method of pregnancy diagnosis to detect non-pregnant animals in order to put them into a new round of estrous synchronization. Palpation per rectum is the most frequent method used for pregnancy diagnosis in dairy cattle after 30 days of breeding/artificial insemination (Roberts, 1971; Momont, 1990; Youngquist, 1997). It is considered that a good practitioner is able to detect pregnant/non-pregnant animals from day 35 on (Euler, 1930; Götze, 1940; Roberts, 1971; Zemjanis, 1971; Momont, 1990).

Information about the potential deleterious effect of palpation per rectum for early pregnancy diagnosis on embryo/fetus viability is contradictory. Some studies have suggested a possible adverse effect of early palpation per rectum (Abbitt *et al*., 1978; Paisley *et al*., 1978; Vaillancourt *et al*., 1979; Franco *et al*., 1987; White *et al*., 1989; McLeod and Williams, 1991). In contrast, other recent studies (Thurmond and Picanso, 1993; Thompson *et al*., 1994) have suggested little effect of the time at which the first palpation per rectum is performed after insemination on calving rate. In summary, all the previous studies have several confounding factors: viability of embryo/fetus was unknown, absence of a control group (non-palpated per rectum group) technique and interval to evaluate the palpation per rectum, differences among farms, differences among categories (cows versus heifers), presence of twins, procedure of palpation per rectum and number of sick/ill females. Previous reports that diagnosed

pregnant females by palpation per rectum (Abbitt *et al*., 1978, Paisley *et al*., 1978; Vaillancourt *et al*., 1979), progesterone (Franco *et al*., 1987) or protein B (Alexander *et al*., 1995) did not assess the viability of the embryo/fetus. In spontaneous or induced embryo/fetal mortality elevated levels of progesterone or protein B (Kassam *et al*., 1987; Maurer *et al*., 1985; Chapter V) as well as positive signs of pregnancy persisted for several days despite the embryo/fetal death (Parmigiani *et al*., 1978; Kassam *et al*., 1987; Chapter V). Most of the studies lack a "pregnant non-palpated group" (control group) (Abbitt *et al*., 1978, Paisley *et al*., 1978; Vaillancourt *et al*., 1979) to differentiate the effects of palpation per rectum from spontaneous embryo/fetal death occurring during early pregnancy. The interval between palpation per rectum and reevaluation was variable: from 30 to 90 days (Abbittt *et al*., 1978), 44 to 48 days (Franco *et al*., 1987), or at calving (Paisley *et al*, 1978) or variable depending if the palpation was performed before or after 40 days of pregnancy (Vaillancourt *et al*., 1979). This is important because the embryo/fetus can be affected by factors other than palpation per rectum. Differences among farms are well established and are more related to management factors than to infectious diseases (Thompson *et al*., 1994). This was not taken into consideration in some studies. Most of the previous reports pool together heifers with cows. One study showed that pregnant heifers have lower embryo/fetal mortality rates than cows (Labernia *et al*., 1996) however, these data were retrospective and with no control group. Previous studies did not report the number of twin pregnancies. Twin pregnancies increase the risk of embryo/fetal death and abortion (Day *et al*., 1995). Moreover, in previous studies, real practice conditions were not followed. For example the animals were palpated per rectum by more than one person at the same time, different techniques were used at the same time, or different techniques were used in the same animal by more than one person (Abbitt *et al*., 1978; Paisley *et al*., 1978; Vaillancourt *et al*., 1979; Franco *et al*., 1987). In the above cases, the procedure of palpation per rectum was more invasive than the one used for diagnosis of pregnancy in practice.

Confirmation of pregnancy status before or at the time of palpation per rectum by another method allows the differentiation of naturally occurring embryonic/fetal loss from embryonic/fetal loss potentially induced by palpation per rectum. The use of transrectal ultrasonography permits an earlier pregnancy diagnosis than palpation per rectum, gives immediate information about the presence of positive signs of pregnancy as well as on embryo/fetus viability and reduces the number of false positive diagnoses and false negatives when palpation per rectum is used (Romano and Magee, 2001). In addition, reports about the use of transrectal ultrasonography have shown that it is a safe technique that does not affect the embryo or fetus viability (Kahn, 1992; Ball and Longue, 1994; Baxter and Ward, 1997).

The objective of the present study was to evaluate the effect of palpation per rectum using the fetal membrane slip technique for early pregnancy diagnosis on embryo/fetus viability in dairy cattle.

MATERIALS AND METHODS

The study was conducted in the Dairy Cattle Center of Texas A & M University. Holstein and Jersey cows and heifers were used. Each cow was inseminated after the voluntary waiting period of 55 days postpartum. Each heifer was inseminated between 14 and 16 months of age. The females were estrous synchronized by using prostaglandin F-2α and artificial inseminated with the am and pm rule (estrus= day 0). Lactating cows were housed in concrete dry lots covered free stalls, milked twice daily, and fed a total mixed diet consisting of corn or grain sorghum, soybean meal, alfalfa, and corn silage. Mineral salt and water were offered ad libitum. Diets were formulated to meet or exceed NRC requirements (1989). The body condition score was evaluated

during all the period of investigation and the scale used was between 1 and 5 (Wildman *et al*., 1982).

All animals were free of tuberculosis and brucellosis. Pregnant cows/heifers that developed clinical mastitis, severe degree of lameness or severe digestive disorders were excluded. The vaccination program included only products that contained killed bacteria and viruses. The time of vaccination for cows was scheduled at the following periods: at post-partum (25-30 days), vaccination 6 months later, at dry-off (-60 days) and pre-partum (-15 days).

The total number of females included in the experimental design are presented in Table 3. Five hundred and twenty pregnant females (360 cows and 160 heifers) with a viable embryo detected by transrectal ultrasonography between days 29 and 32 after artificial insemination were used. A viable embryo was defined as an embryo with heart rate above 120 beats per minute determined by counting the number of contractions/minute or by using M-mode of the ultrasound. The pregnant females were randomly divided in two equals groups: palpation per rectum (PAL group) and no palpation per rectum (NPAL group). The PAL group was submitted to palpation per rectum using the fetal membrane slip (FMS) technique once between days 34 and 41 of pregnancy. The fetal membrane slip was performed in the pregnant horn (ipsilateral to the CL). Palpation per rectum was performed by the same person who avoided palpating the amniotic sac. Throughout the investigation period the females did not undergo any other palpation per rectum. Both groups were submitted to two additional transrectal ultrasonographies at days 45 and 60 of pregnancy. Day 45 was used to monitor the potential immediate deleterious effect of palpation per rectum on embryo viability. Day 60 was used to monitor the potential delayed deleterious effect of palpation per rectum on fetus viability. All transrectal ultrasonographies were performed in the morning, by the same operator, using an Aloka SSD 500 ultrasound machine equipped with a 5 MHz linear transducer. During the transrectal ultrasonography procedure the operator

removed the feces from the rectum, introduced the probe for scanning and avoided grasping the uterine horns. The probe was cleaned between animals. The diagnosis of embryo/fetal death was made when there was no embryo/fetus heart beat, signs of embryo/fetus degeneration were observed or when the positive signs of pregnancy were absent in a cow/heifer previously diagnosed as pregnant.

Table 3. Number of pregnant females included in the controlled randomized block design.

	Total	PAL group	NPAL group
Total animals	520	258	262
By category:			
Cows (%)	360 (69.2)	177 (68.6)	183 (69.9)
Heifers (%)	160 (30.8)	81 (31.4)	79 (30.1)
By number of embryo			
Singles (%)	473 (91.0)	235 (91.1)	238 (90.8)
Twins (%)	47 (9.0)	23 (8.9)	24 (9.2)

The proportion of animals suffering embryo/fetal death were compared between treatment groups using Chi-square analysis with “Yates” correction. A difference was considered statistically significant at $P<0.05$ (Devore and Peck, 1993).

RESULTS

The summary of results is shown in Table 4. The overall embryo/fetal death between days 30 and 60 was 14.0% (73/520). Embryonic death (from 30 to 45 days; 10.0%; 52/520) was significantly higher than fetal death (from 46 to 60 days; 4.5%; 21/468; $P<0.001$). Embryo/fetus death between PAL group (14.7%; 38/258) and NPAL group (13.4%; 35/262) was not significantly different ($P>0.05$). Embryo death between PAL and NPAL groups was 9.3% (24/258) and 10.7% (28/262), respectively ($P>0.05$). Fetus death between PAL and NPAL group was 5.9% (14/234) and 3.0% (7/234), respectively ($P>0.05$). In the PAL group, the embryo mortality from days 34 to 37 was 16.5% (21/127) and from days 38 to 41 was 12.9% (17/131), respectively ($P>0.05$). In cows, embryo/fetus death between PAL (18.6%; 33/177) and NPAL (14.2%; 26/183) was not different ($P>0.05$). In heifers, embryo/fetus death, for the same groups was 6.2% (5/81) and 11.4% (9/79) respectively, which was not different ($P>0.05$). Embryo/fetus mortality was higher in cows (16.4%; 59/360) than in heifers (8.8%; 14/160; $P<0.025$). In single pregnancies the proportion of embryo/fetus localized in the right uterine horn was 61.7% (292/473) and in the left uterine horn was 38.3% (181/473; $P<0.01$). The percentage of twins for all pregnancies was 9.0% (47/52) and increased to 12.8% (46/360) when only cows were included. The percentage of twins for heifers (0.6%; 1/160) was different from the cows (12.8%; 46/360; $P<0.001$). Embryo/fetus mortality was higher in twins (25.5 %; 12/47) than in single pregnancies (12.9%; 61/473; $P<0.025$). The number of twins for PAL and NPAL groups was 23 and 24, respectively. Embryo/fetus mortality for twins was 21.7% (5/23) for PAL group and 29.2% (7/24) for NPAL group, respectively ($P>0.05$).

DISCUSSION

The present study shows that spontaneous embryo/fetal mortality occurs

at a relatively high level in early pregnancy but it is not affected by palpation per rectum using the fetal membrane slip procedure. This study was designed to avoid the potential confounding factors found in previous reports. The factors that were taken into consideration were: 1) only animals pregnant with a viable embryo were included; 2) control group (a non-palpated per rectum group) was included; 3) the animals were submitted to subsequent TRUS evaluation to determine the potential immediate (45 days) and delayed (60 days) effect of PPR; 4) the percentage of animals pregnant with twins was equilibrated between groups; 5) the animals which were sick/ill during the experimental period were eliminated; 6) cows and heifers were analyzed separately and 7) special attention was taken regarding the palpation per rectum procedure used.

Table 4. Embryo/fetal mortality in dairy cow/heifers in palpated per rectum (PAL) and not palpated per rectum (NPAL) groups.

	Total	PAL group	NPAL group	Probability
1-Total (%)	73 (14.0)	38 (14.7)	35 (13.4)	P>0.05
2- By category:				
Cows (%)	59 (16.4)[a]	33 (18.6)[a]	26 (14.2)[a]	P>0.05
Heifers (%)	14 (8.8)[c]	5 (6.2)[d]	9 (11.4)[a]	P>0.05
3- By period:				
Embryo (%)	52 (10.0)[a]	24 (9.3)[a]	28 (10.7)[a]	P>0.05
Fetal (%)	21 (4.5)[e]	14 (5.9)[a]	7 (3.0)[e]	P>0.05
4- By number of embryos:				
Singles (%)	61 (12.9)[a]	33 (14.0)[a]	28 (11.8)[a]	P>0.05
Twins (%)	12 (25.5)[c]	5 (21.7)[a]	7 (29.2)[c]	P>0.05

Different superscripts in the same column are significant: [ab]P<0.05, [ac]P<0.025, [ad]P<0.01, [ae]P<0.001

Several other studies have dealt with the effect of palpation per rectum on embryo/fetal mortality. Some were retrospective (Paisley *et al*., 1978; Vaillancourt *et al*., 1979) others prospective comparing among techniques (Abbitt *et al*., 1978). In all of these studies, however, a non-palpated control was absent. This was possibly, because at the time of these studies were performed a reliable method for pregnancy diagnosis was not available.

Early pregnancy diagnosis was associated with decreased diagnostic accuracy or increased embryo/fetal loss or increased calving interval (White *et al*., 1989), however, there is no consensus (Thompson *et al*., 1995). In a retrospective study, cows diagnosed pregnant by experienced veterinarians, at 41 days or less after insemination were significantly less likely to calve than were cows diagnosed pregnant at a later day (White *et al*., 1989). In Holstein cows from 3 dairies, prenatal losses were 5.8% for cows palpated at less than 35 days, 6% for cows palpated between 35 and 45 days and 0.9% for palpations per rectum later than 45 days after AI (Paisley *et al*., 1978). Among 892 cows from 14 herds, apparent fetal loss was 8.5% for cows palpated 35 to 51 days post breeding and 3.7% if palpation was done at 52 to 70 days estimated by palpation per rectum 30 to 90 days after the initial diagnosis (Abbitt *et al*., 1978). A retrospective study of nearly 7500 Holstein and Guernsey cows found that embryonic loss measured by rebreeding or subsequent palpation per rectum was 7.2% among cows in the first 50 days after breeding compared with 3.2 % for cows palpated more than 50 days after breeding (Vaillancourt *et al*., 1979). All these three studies lack a pregnant non-palpated group; however, all were in agreement that the earlier the pregnancy diagnosis was done the higher the embryo/fetal mortality. In the present study, only pregnant animals with a viable embryo, and a non-palpated group, were included. Thus, we were able to separate the effects of palpation per rectum from the effect of spontaneous embryo/fetal mortality

during early stages of pregnancy. In the non palpated group of our study, the embryo mortality was higher than fetal mortality (10.7% versus 3.0% respectively) and in agreement with some of the reports previously mentioned. Studies that used milk progesterone analysis, as the indication of pregnancy, reported an embryo/fetal mortality of 10 to 28% from days 14 to 70 after insemination (Ball, 1978). A recent study from California showed an embryo/fetal mortality of 19% between days 28 and 90 in lactating dairy cows (Santos *et al*., 2001).

The use of transrectal ultrasonography improved the precision of the experimental design because only animals pregnant with a healthy embryo were included and non-pregnant and pregnant animals with dead embryos were excluded. In addition, pregnant animals that, by palpation per rectum, would have been diagnosed open were included (reduction of false negative; Romano and Magee, 2001). In previous studies, pregnancy diagnosis was assumed to be 100% accurate. In studies that used palpation per rectum as the method of diagnosis the probability of false negative diagnosis was not tested (elimination of true pregnant females) or the inclusion of animals that were really non-pregnant (production of "false" pregnant females) was not ruled out. Some studies have shown that palpation per rectum for pregnancy diagnosis was not an accurate procedure during the early stages of gestation. Two studies reported that 9 % and 5% of the cows diagnosed open calved at a time consistent with being pregnant when the diagnosis was made (Reimers *et al*., 1985; Warnick *et al*., 1995). Another study using only heifers showed that 9% of the animals detected pregnant by protein B at 30 to 45 days were not detected by palpation per rectum (Alexander *et al*., 1995). After spontaneous embryo/fetal death, the presence of positive signs of pregnancy (positive membrane slip) by palpation per rectum persists for some time in an already dead embryo and can not be determined precisely during early stages of embryo

mortality (Romano and Magee, 2001; Chapter V). In the present study, 15 pregnant females detected by TRUS were initially excluded from the experiment due to the presence of an embryo already dead, with positive fetal membrane slip. These animals would have been "false positives" if palpation per rectum, progesterone level or protein B had been used for the initial pregnancy diagnosis.

In the present study 26.3% (10/38) of the females included in the PAL group presented negative signs of pregnancy (absence of fetal membrane slip) at the time of their assigned day of PPR. These animals were further confirmed, the same day, not pregnant by TRUS. This shows clearly that the embryo death was not due to the PPR per se. However, all these animals were not excluded from the statistical analysis. On the other hand, the possibility that pregnant animals with a positive fetal membrane slip at the time of PPR in which the embryo was already dead or in the early process of degeneration was not tested and can not be ruled-out. In spontaneous embryo/fetal death a positive fetal membrane slip persisted from 16.2 days (range 7-28; Chapter V) and similar values of 15.9 days (range 7-27; Ball and Carroll, 1963), 16.3 days (range: 5-50; Dawson, 1974) and 18 days (Kassam *et al*., 1987) were obtained when iatrogenic abortion by amniotic vesicle rupture was performed.

In this study, the palpation technique was probably similar to those used by most veterinarians in private practice, that is, each female is submitted to palpation per rectum by only one person once trying to find a positive fetal membrane slip. In previous studies, realistic conditions were not followed because the animals were evaluated for more than one person at the same time, different techniques were used at the same time, or different techniques were used in the same animal by more than one person (Abbitt *et al*., 1978; Paisley *et al*., 1978; Vaillancourt *et al*., 1979, Franco *et al*., 1987). All these studies showed a more invasive palpation per rectum procedure than the one used in the

present study for pregnancy evaluation. The females studied through University Clinical Teaching Programs may have experienced more trauma as the result of less experienced persons and/or more rigorous and extensive examination of the reproductive tract than what is typical in private practice. It is necessary to remark that palpation per rectum can be used for embryo/fetal attrition and was frequently used before the introduction of prostaglandin F-2α and its potential effect can not be ruled out (Ball and Carroll, 1963; Rowson and Dott, 1963; Dawson, 1974; Parmigiani *et al*., 1978). According to some authors the use of fetal membrane slip can harm embryo/fetuses (Abbitt *et al*., 1978). However, in this last study, that included 2 trials, when the technique was used on one farm by only one trained clinician (first trial) no difference in embryo/fetal mortality was found compared to other techniques (fluctuation and amniotic sac palpation). Older reports consider fetal membrane slip a safe technique for early pregnancy diagnosis (Euler, 1930; Götze 1940). There are few studies comparing different techniques of palpation per rectum for early pregnancy diagnosis. Two of these reports found no difference between the fetal membrane slip and amniotic sac palpation (Studer, 1969; Callahan, 1969). It is necessary to remark that the present study was not designed to explore differences between techniques, because only one technique was used. However, from the results obtained, the fetal membrane slip did not produce damage in the embryo compared to the non-palpated control group.

Herds are affected by a multitude of management factors that influence calving rates (Thompson *et al*., 1994). Other studies have reported a herd effect on the relationship between palpation per rectum and embryo/fetal loss (Thursmond and Picanso, 1993). A significant difference was also found among herds in different years (Vaillancourt *et al*., 1979). Many studies regarding palpation per rectum pulled together

data from different farms. In the present study, only one farm, under a very strong herd health management program, was used.

Field data and review papers have documented large differences among breeds in twinning rate (Rutledge, 1975; Anderson *et al*., 1978). Twinning rate in Hereford and Angus breeds average less than 1%, but in the Holstein breed exceeds 4% (Rutledge, 1975). The induction of twin-calving by one embryo transfer at day 7 after artificial insemination (Sreenan *et al*., 1981) or two embryos after unilateral or bilateral transfer (Rowson *et al*., 1971) produced a high rate of embryo/fetal mortality (Anderson *et al*., 1978). Little information is available regarding embryo/fetal death in spontaneously occurring twins. Previous studies regarding mortality and palpation per rectum did not report the number of twin pregnancies (Abbitt *et al*., 1978; Paisley *et al*., 1978). Factoring twins into the results is important as it can skew the results. In the present study, the number of twins was balanced between groups and the embryo/fetal mortality was twice in comparison to single pregnancies.

Embryo/fetal mortality in cows was almost double that in heifers in the same stage of pregnancy. In most of the studies regarding palpation per rectum, heifers and cows were not separated (Abbitt *et al*., 1978; Paisley *et al*., 1978). A retrospective study using palpation per rectum between 30 to 70 days after breeding showed that embryo/fetal death was almost three times higher in cows than in heifers (Labernia *et al*., 1996). The reasons why cows lose more embryos/fetuses than heifers is unknown. Twinning rate can be one of these factors. In cows which were induced to be pregnant with twins by transferring 2 embryos and then slaughtered at 45 days, the numbers of placentomes were higher in the bilateral transfer than in the unilateral transfer (Rowson *et al*., 1971). Therefore, inadequate nutritional support may be one of the factors. In the present study, the percentage of twin pregnancies in heifers was 0.6% and in cows was

12.8%. When the numbers of cows with twins were excluded from the statistical analysis a reduction in the percentage of embryo/fetal mortality was observed. However, embryo/fetal mortality continued to be higher in cows than heifers. Therefore, factors other than twin pregnancy are implicated in such embryo/fetal mortality. Stress of lactation, inadequate genotypes, deficient nutritional support and insufficient hormonal levels, could account for this increased mortality rate.

In previous studies the interval between palpation per rectum and reevaluation was 30 to 90 days (Abbittt *et al*., 1978), 44 to 48 days (Franco *et al*., 1987), at calving (Paisley *et al*, 1978) or variable depending if the palpation was performed in females before or after 40 days of pregnancy (Vaillancourt *et al*., 1979). In the present study, all the females were scheduled for TRUS at 45 and 60 days of pregnancy. The rationale was to maintain an identical interval sufficient enough to determine the true association between palpation per rectum and embryo/fetal mortality and to exclude factors other than palpation per rectum that could affect pregnancy. The goal of the TRUS at 45 days was to determine an immediate effect of palpation per rectum during the embryo period and the goal of the TRUS at 60 days was to determine a delayed effect of palpation per rectum manifested during the fetal period (from 46 to 60 days).

CHAPTER IV

SPONTANEOUS EMBRYO/FETAL MORTALITY IN DAIRY CATTLE

INTRODUCTION

Embryonic/fetal mortality is the most important factor that reduces the reproductive efficiency in cattle (Ayalon, 1978; Wilmut and Sales, 1981; Peters, 1996). The importance of this loss is not only technical but also economical (Thurmond and Picanso, 1993). In modern dairy practice lactating cows continue to experience a high level of pregnancy loss, despite intensive efforts towards reducing specific infectious diseases through preventive heath programs that include strict protocols of immunization against specific pathogenic agents and other sanitary measures. The endemic nature of this loss suggests that factors other than or in addition to pathogens may contribute to the risk of abortion (Thurmond *et al*., 1990; Forar *et al*., 1995). Little is known about late spontaneous embryo/fetal mortality in cattle and few studies provide a description of the process (Kastelic *et al*., 1991; Forar *et al*., 1995). In cattle, based on anecdotal observations apparently two types of spontaneous embryo/fetal mortality based on palpation per rectum and ultrasonographic images of the genital tract exist (JE Romano, unpublished observations). Moreover, in *in vitro* produced bovine embryos, especially in cloned embryos, embryo/fetal mortality during the first trimester of pregnancy is much higher than in *in vivo* produced pregnancies. This high embryo/fetal mortality is currently reducing the efficiency of this new reproductive biotechnology (Hill *et al*., 2000).

Factors that were found to influence embryo/fetus mortality are multiple. Nevertheless, despite many years of study, the causes of reproductive failure in farm animals are poorly understood. Most investigations have underestimated the incidence of pregnancy loss, and few investigators have attempted to identify when during gestation the conceptus dies and the causes of death. Searching for answers, the pathologist has

looked for infection; the endocrinologist for hormonal imbalances, the geneticist for chromosomal aberrations, and so on through the various associated disciplines. Each search has usually yielded some suggestion of a possible physiological or pathological cause of embryo/fetal death without any definitive answer. Until a greater understanding of embryo/fetal mortality is reached, little can be done to improve the reproductive efficiency, design preventive measures and treatments and prevent economic loss.

The present study was designed to improve some deficiencies of previous studies related to pregnancy loss by using early, regular and repeated TRUS. Early detection of pregnancy in all females at the same intervals permits a sound comparison among groups and times. The use of transrectal ultrasonography adds information about the viability of the embryo/fetus, reduces false positive and permits the inclusion of false negatives (information that is not included when palpation per rectum is the only method of pregnancy diagnosis in this early stage) (Romano and Magee, 2001). Moreover, the possibility of diagnosing as pregnant a female with positive signs of pregnancy by palpation per rectum (with a dead embryo) was eliminated by the use of TRUS (Chapter III). Also, TRUS offers a more precise time of embryo/fetus death. In addition, reports about the use of transrectal ultrasonography have shown that it is a safe technique that does not affect the embryo or fetus viability (Kahn, 1992; Ball and Longue, 1994; Baxter and Ward, 1997).

The present observational study was designed to evaluate several factors on spontaneous embryo/fetal mortality from days 30 to 120 of gestation in dairy cattle.

MATERIAL AND METHODS

The study was conducted in the Dairy Cattle Center of Texas A & M University between 1999 and 2003. Holstein and Jersey cows and heifers were used. Each cow was inseminated after a voluntary waiting period of 55 days postpartum. Each heifer was inseminated between 14 and 16 months of age. The females were artificially inseminated after natural estrus or estrous synchronization using prostaglandin F-2α. All females were estrus detected twice or thrice a day and artificially inseminated based on the am-

pm rule (Dransfield *et al*., 1998). No pregnancies from artificial insemination at fixed times were included. Lactating cows were housed in concrete dry lots, covered free stalls, milked twice daily, and fed a total mixed diet consisting of corn or grain sorghum, soybean meal, alfalfa, corn silage and high quality hay offered twice a day. Mineral salt and water were offered at libitum. Diets were formulated to meet or exceed NRC recommendations (1989). The body condition score was evaluated in the pregnant animals throughout the study period using a scale of 1 to 5 (Wildman *et al*., 1982). In late spring and summer the animals were maintained in a shaded area, with sprinklers and fans in order to reduce the effect of temperature and humidity (Thompson *et al*., 1999). The herd ranged from 115 to 125 milking cows per year, and had a rolling average annual milk production of 19,300 pounds with an average annual somatic cell count below 280,000.

All females between 25-30 days postpartum were evaluated for uterine involution/pathology, ovarian activity and body condition score. At this time they were vaccinated with *Escherichia coli* bacterin-toxoid (J-5; Merial Laboratories), Infectious Bovine Rhinotracheitis, Bovine Viral Diarrhea types 1 and 2, Parainfluenza 3, Bovine Respiratory Syncytial Virus Vaccine, Killed virus- *Campylobacter fetus* and *Leptospira canicola, grippotyphosa, hardjo, icterohaemorrhagiae, pomona* bacterin (Virashield 5 + 5VL; Grand Laboratories, Freeman, SD 57029). At seven months of pregnancy the females were rechecked by palpation per rectum, treated with dry intra-mammary infusion of antibiotics according to the mastitis record, evaluated for Body Condition Score and given Clostridium vaccine (*Clostridium chauvoei, septicum, haemolyticum, novyi, sordelli-perfringens* types C & D bacterin-toxoid; Ultrabac 8, Pfizer Animal Health), *Escherichia Coli* bacterin-toxoid and *Salmonella typhimurium* bacterin-toxoid plus immune stimulator (Endovac-Bovi with immuneplus; Immvac, Columbia, MO 65021). Pregnant heifers underwent the same procedure except for the mammary treatment. At 260-265 days the females were moved to the calving pasture. At this time they were vaccinated again with *Salmonella typhimurium* bacterin-toxoid plus immune stimulator, Bovine Rota-Coronovirus and *Escherichia Coli* bacterin-toxoid (Scourguard

3K; Pfizer Animal Health) and Virashield 5 VL 5. The female calves were vaccinated against Clostridium at 6, 8, 12 and 24 weeks and with Virashield 5 VL5 at 1.5, 3, 6 and 12 and 14 months of age. The management system was “all in and all out”. New animals introduced in the herd came from farms with specific sanitary programs including specific tests performed. All frozen-thawed semen was from Certified Semen Services (CSS). The herd was free from tuberculosis, brucellosis and bovine leukemia virus. The sera-prevalence for Johne’s disease positive animals had been less than 5% for the previous 4 years. In the event of a detected abortion, the placenta/fetus was sent immediately to Texas Veterinary Medical Diagnostic Lab in order to test for infectious diseases.

Five hundred and fifty one pregnancies (394 in cows and 157 in heifers) with a viable embryo detected by transrectal ultrasonography between days 29 and 32 after artificial insemination were studied. The numbers of pregnant cows in their first, second, third, fourth and fifth or more lactations were 159, 119, 67, 31 and 18, respectively. A viable embryo was defined as an embryo with heart rate above 120 beats per minute which was determined by counting the number of contractions/minute or by using M-mode of the ultrasound. Each pregnant female was scheduled for further TRUS at days 45, 60, 75 and 120 days of pregnancy. All transrectal ultrasonographies were performed in the morning, by the same operator, using an Aloka 500 V ultrasound machine equipped with a 5 MHz linear transducer. During the procedure the operator removed the feces from the rectum and introduced the probe for scanning the reproductive tract. The transducer was cleaned between animals by using a dilute solution of antiseptic. The diagnosis of embryo/fetal death was made when there was no embryonic/fetal heart beat, signs of embryo/fetus degeneration were observed or when the positive signs of pregnancy were absent in a cow/heifer previously diagnosed as pregnant. In advanced stages of pregnancy, viability of the fetus was considered when spontaneous or induced movements as well as pulsation of the umbilical cord were noted. As defined for this study, the embryonic period extends from pregnancy detection to completion of the stage of organogenesis (approximately 45 days). The fetal period extends from the time

differentiation is completed to parturition (from >45 days). Embryonic mortality was defined as the death or loss of the conceptus during the embryonic period. Late embryonic mortality was the embryonic death from the time of pregnancy diagnosis until day 45. Fetal mortality was the death or loss of the conceptus during the fetal period. Embryonic/fetal mortality (also pregnancy loss) included the death or loss of the conceptus during both embryonic and fetal periods. Clinical observations in practice show two types of spontaneous embryo/fetal mortality in cattle based in the palpation per rectum and ultrasonographic images of the embryo/fetus (JE Romano, unpublished observations). The criteria used to classify these two types of embryo/fetal mortality were presence/absence of embryo/fetus degeneration, presence/absence of positive signs of pregnancy (presence of fetal membrane slip) and presence/absence of a functional corpus luteum at the time of palpation per rectum/transrectal ultrasonography. These two types of embryo/fetal mortality were named as Type I and Type II. Type I was characterized by a positive fetal membrane slip by palpation per rectum, presence of a degenerated embryo/fetus and a functional corpus luteum by transrectal ultrasonography. Type II was characterized by the absence of an embryo/fetus, absence of positive membrane fetal slip in a female previously diagnosed pregnant with a live embryo. In some cases, a corpus luteum was seen. Type II was characterized as an empty uterus in a female previously diagnosed as pregnant.

Cox Proportional Hazards analysis model that includes the following variables of embryo/fetal mortality was used: 1) age; 2) lactation number; 3) period of embryo/fetal death; 4) type of embryo/fetal mortality and 5) singles versus twin pregnancies. Also, the proportions of females with embryo/fetus mortality in relation to the total females pregnant were calculated and compared among periods, age, lactation number, types of embryo/fetal mortality and single versus twin pregnancy using the Likelihood ratio Chi-square analysis. A difference was considered statistically significant at $P<0.05$.

RESULTS

The overall embryo/fetal mortality in the first four months of pregnancy was 19.2% (106/551). Late embryo mortality (10.9%) was higher than fetal mortality ($P<0.001$). The overall fetal mortality for periods 46 to 60, 61 to 75 and 76 to 120 days was 4.3%, 2.3% and 3.1% respectively ($P>0.05$; see Figure 1). Embryo/fetal mortality in single pregnancies (16.9%; 84/497) was lower than in twin pregnancies (40.7%; 22/54; $P<0.001$; see Figure 2). In single pregnancies, embryo/fetal mortality for periods 30 to 45, 46 to 60, 61 to 75 and 76 to 120 was 10.9%, 2.9%, 1.6% and 2.3%, respectively ($P<0.001$). In twin pregnancies for the same periods it was 11.1%, 16.7%, 10% and 11.1%, respectively ($P>0.05$). Late embryo mortality between single and twin pregnancies was not different ($P>0.05$), however, during the three fetal periods a different distribution between single and twin pregnancies was detected ($P<0.01$).

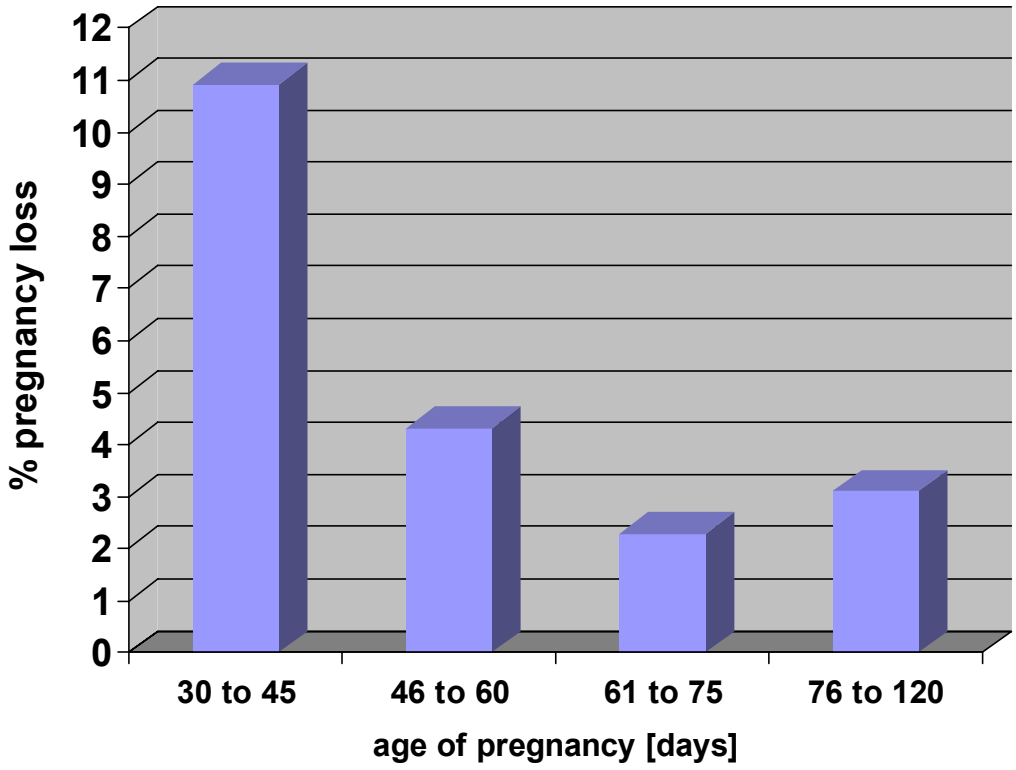

Figure 1. Results from spontaneous embryo/fetus mortality by period.

In single pregnancies, embryo/fetal mortality Type I and II was 41.7% and 58.3%, respectively ($P>0.05$). In twin pregnancies, embryo/fetal mortality Type I and II was 72.7% and 27.3%, respectively ($P<0.05$). The embryo/fetal mortality increased from 2 to 3 years-old (12.4% to 21.8%; $P<0.01$) and then was maintained around 22.8% from 3 to 9 years-old. Embryo/fetal mortality in heifers (12.7%) was lower than in cows 21.9% ($P<0.025$) and no differences between first and fourth lactations were detected ($P>0.05$; see Figure 3). Twin pregnancies increased with lactation number being 1.3%, 8.2%, 14.3%, 19.4% and 25.8% for heifers, first, second, third and fourth lactations, respectively ($P<0.01$; see Figure 4). Twin pregnancies also increased gradually with the female's age ($P<0.01$). It increased from heifers up to 5 year-old cows, remained steady until 8 year-olds and finally dropped after 9 years of age.

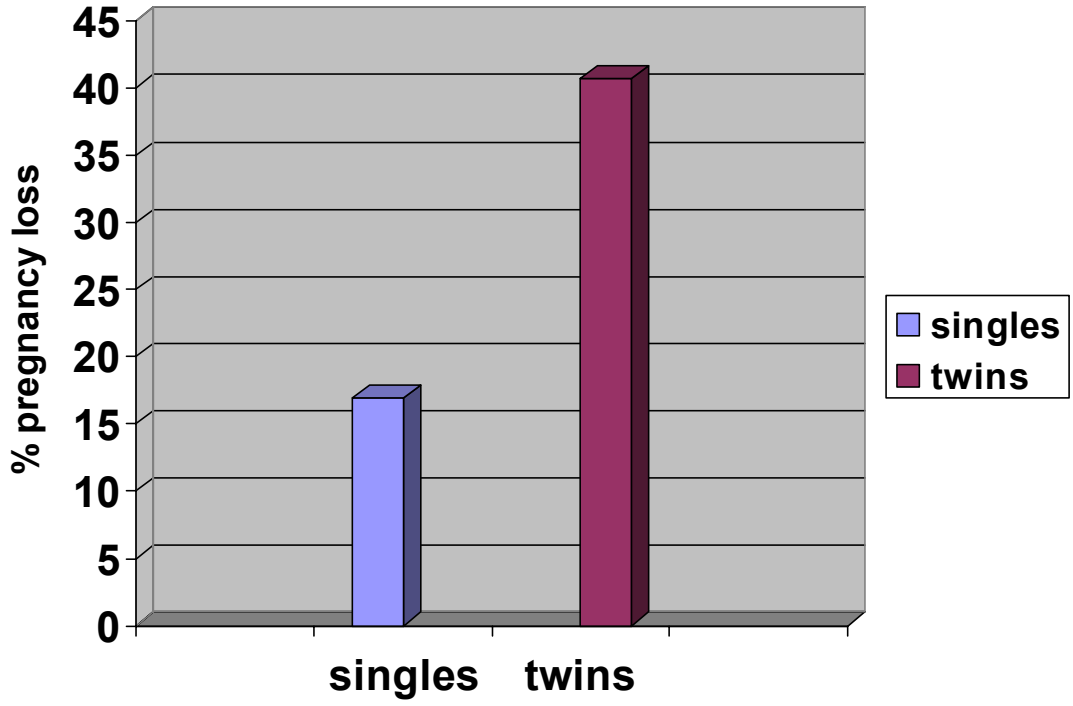

Figure 2. Results from spontaneous embryo/fetus mortality: singles versus twins.

DISCUSSION

The present study was performed on a single dairy farm to ensure that all animals were subjected to the same management practices eliminating the farm effect. Moreover, this farm had a strict herd health program and was under the direct supervision of the author. The farm effect is a well reported finding in pregnancy loss (Thurmond and Picanso, 1990; Thompson *et al*., 1994) and can be due to single or multiple conditions such as nutritional, sanitary and reproductive managerial factors. Forar *et al*., 1995, reviewed 26 studies of embryo/fetal mortality during five decades and found tremendous variations in pregnancy loss among farms. Study populations, definition of pregnancy loss, experimental design and analysis of pregnancy loss appeared to be important sources of variation among studies. Other variables included: herd management, methods of pregnancy and non-pregnancy diagnosis, variability of the risk period not only in the same study but also in the same study population, intervals used for

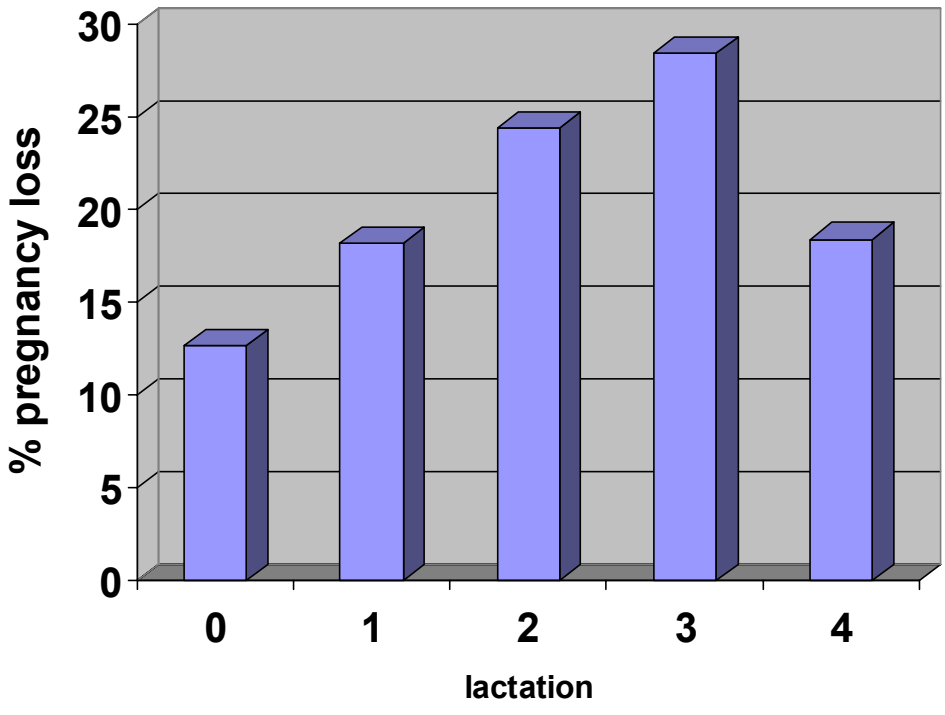

Figure 3. Results from spontaneous embryo/fetus mortality by lactation number.

reexamination, different breeds and herd health protocols. All these confounding factors and their interactions make it very difficult to compare, interpret and arrive at sound conclusions.

In the present study, embryo/fetus mortality was studied by using transrectal ultrasonography and then subjecting the same animals to subsequent and repeated TRUS at predetermined intervals throughout the first half of pregnancy. This approach allowed us to determine the highest period of risk of pregnancy loss and to quantify it. Previous studies, estimated embryo/fetus mortality by slaughtering animals, non-return rates, determining progesterone in milk or blood, presence of protein B in blood, palpation per rectum and in recent years by using ultrasonography. The slaughter of bred females and then flushing their reproductive tract at different intervals from breeding was used to

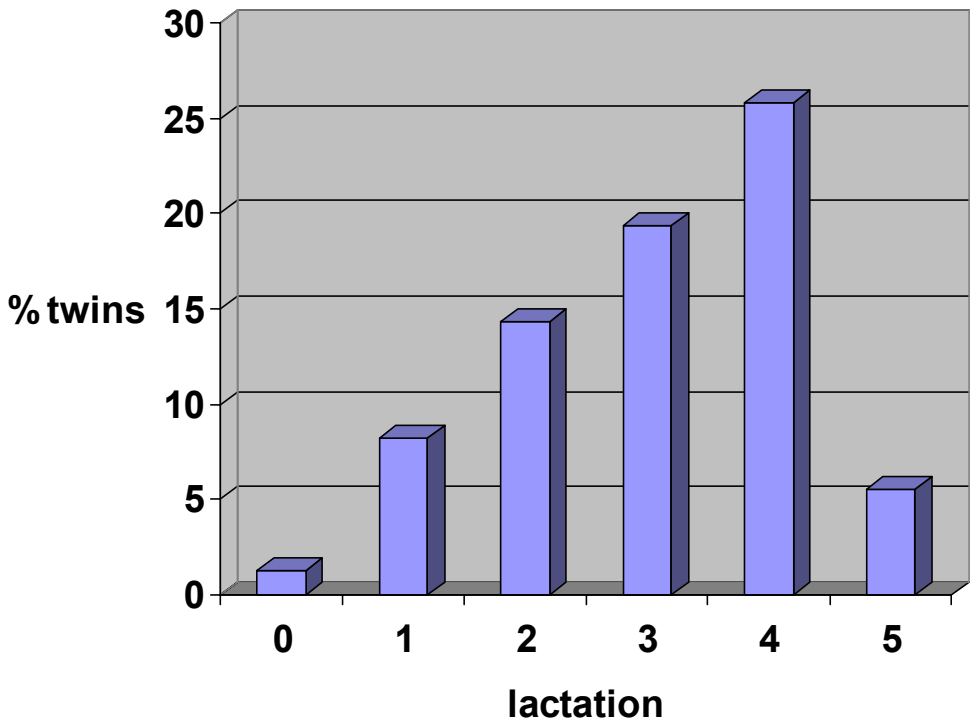

Figure 4. Relation between twin pregnancy and lactation number.

estimate the fertilization rate, early embryo death and late embryo death (Hawk *et al*., 1955b; Diskin and Sreenan, 1980; Maurer and Chenault, 1983). This terminal approach is very precise but each animal contributes only one observation and has an inherent high cost. Non-return rates over-estimate true pregnancy diagnosis (Kidder *et al*., 1954) and are also affected by the quantity and quality of the estrus detection procedure used (Foote, 1974). At present, lactating dairy cows seem to have a lower reproductive performance (Lucy, 2001). Estrus seems to be reduced in intensity and duration in comparison to previous years (Dransfield *et al*.,1998). Therefore, the use of return rates by observing estrus behavior twice a day increases the probability of misdiagnosis. High progesterone levels in milk or blood is considered a better indication of ovarian activity than pregnancy status (Britt, 1995). High levels of progesterone are seen in conditions other than pregnancy such as: inflammation of the uterus, luteal cysts, collection of samples during the luteal phase and in presence of an already dead embryo/fetus in the uterus (Pennington *et al*., 1976; Kassam *et al*., 1987; Chapter V). On the other hand, a low level of progesterone in blood or milk is a good indicator of non-pregnancy, therefore, the level of this hormone in blood or milk is a better indicator of non-pregnancy than of pregnancy (Shemesh *et al*., 1978; Laing *et al*., 1980). Protein B is a glycoprotein hormone produced by the giant cells of the trophoblast that appears in blood after day 15 and consistently from days 24 to 28 after breeding (Sasser *et al*., 1986). However, the maximum sensitivity was obtained at day 37/38 (Szenci *et al*., 1998) or later (Humblot *et al*., 1988a). Another hormone produced by these trophoblastic cells: is glycoprotein associate protein 1 that also appears in peripheral blood. However, its maximum sensitivity was obtained later than for protein B using the same blood samples tested for protein B (Szenci *et al*., 1998). In the case of an embryo/fetus already dead or in the process of degenerating, these hormones remaind elevated in blood for a certain time (Maurer *et al*., 1985; Humblot *et al*., 1988b; Chapter V and VI). Diagnosis of pregnancy by palpation per rectum is a method that is based on the detection of positive signs (allantochorion membrane, amniotic sac, placentomes and fetus) inside the uterus. (Zemjanis, 1971). These signs appear at different intervals from breeding to

examination (Zemjanis, 1971). Definitive information about accuracy of pregnancy diagnosis by palpation per rectum are lacking, however, some authors suggest that is a very accurate method for a trained veterinarian after day 35 post-breeding (Euler, 1930; Götze, 1940; Roberts, 1971; Zemjanis, 1971; Momont, 1990). Some studies have shown that palpation per rectum for pregnancy diagnosis was not an accurate procedure during early stages of gestation. For example, two studies reported that 9 % and 5% of the cows diagnosed open calved at a time consistent with being pregnant when the diagnosis was made (Reimers *et al*., 1985; Warnick *et al*., 1995). Moreover, palpation per rectum during early stages of pregnancy does not provide any information about the viability of the embryo/fetus (Romano and Magee, 2001). Therefore, some animals with an already dead embryo/fetus or in the process of degeneration might show positive signs of pregnancy and will later be found open or will not calve when expected (Chapters III and IV). Spontaneous late embryo mortality is known to be higher than fetal mortality (Chapter IV), therefore, the proportion of animals detected as pregnant that later will be found open is higher the earlier the pregnancy is diagnosed. In recent years, transrectal ultrasonography was used to detect early pregnancy and determine embryo/fetal death (Vasconcelos, 1997; Santos, 2001). Transrectal ultrasonography for pregnancy diagnosis offers some advantages over palpation per rectum: earlier diagnosis of pregnancy/non-pregnancy, determination of embryo/fetus viability, reduction of misdiagnosis (false negatives and false positives) and reduction of "potential" iatrogenic embryo/fetus attrition (Romano and Magee, 2001). In addition, several reports have shown it to be a safe technique with no effect on embryo/fetus viability (Kahn, 1992; Ball and Longue, 1994; Baxter and Ward, 1997). In a recent study, the maximum sensitivity and negative predictive values were obtained from day 29 on in cows and from day 26 on in heifers (Chapter II).

In this study, each animal was evaluated in repeated opportunities at regular intervals with allows for a more exact determination of when embryo/fetal death occurs. The interval used was short enough to permit detecting when death occurred without increasing the stress in the evaluated females. Previous studies had problems

establishing exact time of death of the conceptus. Unobserved return to estrus, delays between the death of the conceptus and its abortion are important factors that make it difficult to determine an accurate time of death. Hawk *et al*., (1955a) reported that 87% (39/45) of the pregnancy losses were unobserved in the first 150 days of pregnancy. Observing 10 Holstein dairies, it was determined that there was a total pregnancy loss of 20%, however, only 8.9% (15/167) were detected in the first 125 days of pregnancy (Forar *et al*., 1996). Estrus detection efficiency is very low, therefore, the possibility of missing estrus is very high (Foote, 1974; Dransfield *et al*., 1998). Also, return to estrus is not a definite criterion of pregnancy loss. Pregnant cows show estrus from 4 to 15% during the first trimester of gestation (Donald, 1943; Donoho and Rickard, 1955; Gilmore, 1952). The insemination of confirmed pregnant cows resulted in embryo/fetus death (Weaver *et al*., 1989; Sturman *et al*, 2000) with abortion some weeks later (Dawson, 1974). Therefore, studies that based the pregnancy loss on return to estrus or abortion in pregnant females have inherent deficiencies.

The number of embryos is an important factor that affects embryo/fetus mortality. Twin pregnancies have more than double the risk of abortion compared to single pregnancies. These results are in agreement with two previous reports. However, one of these studies used palpation per rectum between days 30 and 70 of pregnancy (Day *et al*., 1995) while the other study used transrectal ultrasonography between 38 to 44 days (López-Gatius *et al*., 2002) as methods for pregnancy diagnosis. These reports found that the risk of pregnancy loss for twins was 2.8 times and 3.1 times greater than for single pregnancies, respectively. In single pregnancies, late embryo mortality was higher than fetal mortality. This corresponds to the period in which the allantochorion villus (cotyledons) attaches to the crypts in the endometrium (King *et al*., 1979 and 1982). The distribution and type of embryo/fetal mortality was different between single and twin pregnancies. Late embryo mortality was as high in twin pregnancies as in single pregnancies but remained elevated throughout the three fetal periods (46-60, 61-75 and 76-120). Another interesting finding was that the majority of the cases of embryo/fetus mortality noted in twins were Type I. Competition between embryos in

twin pregnancy for nutrition, space or both could account for some of embryo/fetus loses. The induction of twin-calving by one embryo transfer at day 7 after artificial insemination (Sreenan *et al*.,1981) or two embryos after unilateral or bilateral transfer (Rowson *et al*., 1971) produced a higher rate of embryo/fetal mortality (Anderson *et al*., 1978). A higher survival rate was noted for embryos transferred bilaterally (Rowson *et al*., 1971; Sreenan and Beehan, 1976). Moreover, the number of placentomes was higher in bilateral than unilateral transfer in females slaughtered at 60-90 days of pregnancy (Rowson *et al*., 1971). Most of the previous studies dealing with pregnancy loss in lactating dairy cows used palpation per rectum to determine pregnancy. In newer reports transrectal ultrasonography was the procedure used. Unfortunately in most of these studies the proportion of females with twin pregnancies was not mentioned. This is an important variable that needs to be taken into consideration during the study of pregnancy loss as twining increases the risk of abortion and therefore can affect the outcome of a "specific" treatment. In a previous study, using fetal membrane slip for pregnancy diagnosis, the separate analysis of pregnant animals with twins from singles (blocking by number of embryos) showed to be a very valuable approach (Chapter III).

In the present study, the proportion of twin pregnancies increased with lactation number and age. The incidence of twinning increased until 5 years then remained steady till 9-years and dropped thereafter. Therefore, the proportion of mature cows will affect the embryo/fetus death of the herd. It is necessary to point out that our study population was formed only by pregnant females. Therefore, the proportion of older cows included in a herd might be a factor that "potentially" reduces the "fertility" of the herd. The removal of twin pregnancies from the statistical analysis reduced the proportion of pregnant cows that lost fetuses at the end of the study. Nevertheless, differences persisted in comparison with the fertility of the heifers. Lactating cows have a higher mortality rate probably due to the stress of lactation, nutritional requirements and hormonal imbalances. The proportion of twin pregnancies among farms can be one of the factors involved in the "farm effect", described in several studies (Thurmond and Picanso, 1990; Thompson *et al*., 1994). It is necessary to state that the number of

lactating cows in the third or later lactations is gradually reduced and that it is affected by different "farm" policies for culling. In general, only healthy animals, with good milk production and reproductive performances are kept. In the present study our population included a selected group of animals throughout the time; therefore, the increase in twins with ageing might not be a real situation. More studies are required to determine if there is a true association between twins, lactation and age. Some reports suggested that milk production increases the twinning rate in lactating cows in Wisconsin (Fricke and Wiltbank, 1999) however, another study from California (Santos *et al*., 2001) did not find an association using the same methods of analysis used in the first report. More research in this area is required.

In the present study, pregnancy loss was lower in heifers than in cows, a fact that is in agreement with a retrospective study from Spain. However, the percentage of pregnancy loss reported was different in those studies compared to this (9.6% for cows and 2.8% for heifers). One factor contributing to this could be that cows began to be evaluated by palpation per rectum between 30 to 70 days after breeding and then were rechecked at 120 to 150 days (Labernia *et al*., 1996). In Israel, similar trends between Holstein cows and heifers were reported (Markusfeld, 1997). Age of the cow was not a risk factor for pregnancy loss in a study from California (Thurmond and Picanso, 1990). Nevertheless, the same author in another study (Thurmond and Picanso, 1990) found the highest rate of abortion to be associated with cows over 8-years old. Markusfeld (1997) suggested that the risk of pregnancy loss might be related to different degrees of immunity in association with age. Ball (1978), reported a gradual increase in the level of pregnancy loss, as estimated by using milk progesterone profiles, particularly beyond the fourth lactation. In the present report, the study population included only females detected pregnant by transrectal ultrasonography around day 30 and did not include the animals that returned to estrus before or were detected open at pregnancy diagnosis. Our results disagree with the report of Erb and Holtz (1958) in which heifers had higher late embryo loss than cows. The reason for that result is difficult to explain. In the present study, heifers were raised on the same Dairy Unit but kept in a separate group until

seven months of pregnancy when they were moved in with all the dry-cows. In lactating cows, the risk of pregnancy loss was similar regardless of parity and in agreement with the results reported in Holstein cows in Israel (Markusfeld, 1997). Higher rates of late embryo/fetus mortality in older females compared with younger ones has been reported in many species (Donaldson, 1984; Brinsko *et al*., 1994; Ball *et al*., 1989). One of the causes could be the inability of the uterus to ensure adequate attachment as the age increases (Donaldson, 1984; Brinsko *et al*., 1994). It is necessary to remark that in this farm, as well as in the present dairy industry, in order for a cow to be maintained in the production unit it needs to be strong enough not only to produce high levels of milk but also to reproduce satisfactorily. To meet these requirements the population is selected in the course of their reproductive/productive life. Therefore, these "old" cows might not represent an average animal.

The present result confirms our previous observations that there are two clinically different types of spontaneous embryo/fetal mortality in cattle. Type I is characterized by a positive fetal membrane slip, an embryo/fetus dead or in the process of degeneration and persistence of a functional corpus luteum. Type II, is characterized by absence of the fetal membrane slip, no signs of embryo/fetus degeneration, a clean uterus and in most cases a non-functional corpus luteum. In embryo/fetal mortality Type I the embryo/fetus is already dead and the corpus luteum continues to produce progesterone until the communication between embryo/fetus-uterus is disrupted and luteolysis occurrs. In embryo/fetus mortality Type II, the inverse seems to occur: the embryo/fetus is observed alive while the uterus does not recognize the presence of an embryo/fetus inside. In the case of embryo/fetus mortality Type I, the fetal membrane slip persists for around two weeks, the uterus requires almost 3 weeks for cleaning and the females show estrus almost one month after the death of the embryo/fetus (Chapter V). In single pregnancies the proportion of embryo/fetal mortality Type I and II was almost similar throughout the study period. However, in twin pregnancies the proportion of Type I was signficatively higher than Type II. Therefore, the presence of embryo/fetus mortality Type I has great clinical importance for several reasons: these

animals will be diagnosed as pregnant by palpation per rectum (during the early stage of embryo/fetus death) and will either be found open in a future examination or will not calve at the predicted date. Second, the farmer/owner can interpret these findings as: either the procedure of palpation per rectum caused abortion (Momont, 1990) or that there was an incorrect diagnosis of pregnancy. Third, the precise time when the embryo/fetus death occurred can not be determined. Four, the risk of "false misdiagnosis" is higher in twin pregnancies than single pregnancies because embryo/fetus mortality Type I was significantly more prevalent. It is necessary to remark that the last interval studied (from days 76 to 120) was a long period (45 days) and may not represent the true incidence of the 2 types of embryo/fetal mortality described. The reason is supported from results previously mentioned. For example, if a female presented after day 75 of TRUS examination with embryo/fetus mortality Type I it is probable that all signs of pregnancy would be negative when examined at day 120. This will make us erroneously diagnose it as embryo/fetal mortality Type II when in reality it was a Type I. Further studies reducing this interval are required to obtain a correct answer to this point.

The possibility of detecting pregnancy loss decreases as the interval between breeding and pregnancy diagnosis increases. In general, for five females detected pregnant at approximately day 30 almost four will have a viable fetus at day 120 of pregnancy. In previous studies, herd sizes influenced both frequency of visits and age in which the pregnancy was diagnosed. As frequency of visits increased, gestational age of the diagnosed pregnancies decreased (Lemire *et al*., 1993). Therefore, the likelihood of finding pregnancy loss is higher in a large herd size compared to a small herd. This study supports previous results that the interval from breeding until a cow is diagnosed open has a significant positive association with the calving interval (Warnick *et al*., 1995). Cows first diagnosed pregnant at day 41 or less were significantly less likely to subsequently have a calf than cows diagnosed later (White *et al*., 1989). Another study also showed higher rates of pregnancy loss if pregnancy diagnosis was done before day 48 of pregnancy (Lemire *et al*., 1993). In Holstein cows from 3 dairies, prenatal losses

were 5.8% for cows palpated at less than 35 days, 6% for cows palpated between 35 and 45 days and 0.9% for palpations per rectum after 45 days post-AI (Paisley *et al*., 1978). Among 892 cows from 14 herds, apparent fetal loss (determined by a second palpation per rectum 30 to 90 days after the initial diagnostic procedure), was 8.5% for cows palpated between days 35 to 51 post breeding and 3.7% for cows palpated between days 52 to 70 (Abbitt *et al*., 1978). A retrospective study of nearly 7500 Holstein and Guernsey cows found that embryonic loss measured by rebreeding or subsequent palpation per rectum was 7.2% among cows in the first 50 days after breeding compared with 3.2 % for cows palpated after 50 days post-breeding (Vaillancourt *et al*., 1979). Recent studies, involving only lactating cows, reported an increase in the percentage of pregnancy loss using transrectal ultrasonography. A California study reported an embryo/fetus mortality of 19% when diagnosis was performed between days 28 and day 90 (Santos *et al*., 2001). Another study, involving herds from central Utah and California, using artificial insemination at fixed time reported 24% of pregnancy loss between days 28 to day 98 (Vasconcelos *et al*., 1997).

In the present study, the pregnancy loss per confirmed pregnancy was 19.2%, higher than the 6.5% calculated in a review of 26 studies from six countries during five decades (Forar *et al*., 1995). However, in this previous review the definitions of pregnancy loss were inconsistent, gestational at risk periods were different, the procedures used to evaluate pregnancy and pregnancy loss made comparisons among studies very difficult. In California, pregnancy loss per confirmed pregnancy with similar risk periods were 10.6% and 8.4% in studies involving 4,732 and 19,411 cows, respectively (Thurmond and Picanso, 1990; Thurmond *et al.,*1990). In a Canadian study which included 4135 cows from 71 Canadian herds, the rate was lower (5.2%), but periods of risk started at day 38 and ended at day 104 (mean 51 days) post breeding (Lemire *et al*., 1993). In Israel, a pregnancy loss of 9.3% was reported when the risk period began between 40 to 50 days after breeding (Markusfeld, 1997). It is necessary to stress that palpation per rectum for pregnancy diagnosis was used in all these studies. Fortunately, new information using transrectal ultrasonographies is available. Our results

of late embryo mortality are in agreement with previous reports that showed approximately 10% of late embryo mortality when detected between days 28-30 and 42-45 days using transrectal ultrasonography (Vasconcellos *et al*., 1998; Santos *et al*., 2001) or by determining progesterone levels in milk (Ball, 1978). However, the number of twin pregnancies was not reported in the first study and in the second study pregnancies were reevaluated by palpation per rectum. Our results are in complete disagreement with the report of Fosgate and Smith (1954) that showed embryo/fetus loss was lower in pregnancies diagnosed between days 34-50 and 90 (1.88%) compared with further intervals. Our results of late embryo mortality in dairy cattle were lower than those reported by Smith and Stevenson (1995) and Cartmill *et al*. (2001) of 18.8% and 24%, respectively. Factors that can be responsible for these differences might be the method of reevaluation of pregnant females (palpation per rectum was used) and the interval after pregnancy diagnosis (10 to 26 days after detection). This interval not only included animals with pregnancy loss during the embryonic period but during the early fetal period as well. As it was shown in the present study, this is a critical period in which the allantochorionic placenta is established. Another recent study reported a 17.8% embryo loss between days 28 to 42 (Chebel *et al*., 2003). In this study, a combination of artificial inseminations at fixed time and detection of estrus was used. Pursley *et al*., (1998) reported an increase in pregnancy loss when artificial insemination at fixed time was used. Pregnancy loss was 9%, 21% and 32% when the time between the second injection of GnRH and artificial insemination was 0 hours, between 8 and 24 hours and 32 hours, respectively. In the present study, artificial insemination at estrus detection was used and the embryo/fetus mortality was the same as another study that used fixed time artificial insemination (Vasconcellos *et al*., 1997). More studies are required to clarify this point.

There are very wide gaps in our knowledge regarding etiological factors and mechanisms by which embryo/fetus death occurs. Our present state of knowledge suggests that embryo/fetal mortality may be the result of multiple causes operating through more than one mechanism. It is evident that our increase in knowledge about these processes can take place only following much more detailed observation,

examination, hormonal profiling of the “natural” occurring event. More studies are required to determine if these two types of embryo/fetal mortality represent really different expressions of the same process or are only one type. More studies increasing the frequency of examination will permit a more precise result.

CHAPTER V

CLINICAL, BEHAVIORAL AND ULTRASONOGRAPHIC CHARACTERISTICS OF FEMALES WITH SPONTANEOUS EMBRYO/FETAL MORTALITY

INTRODUCTION

Embryonic/fetal mortality has a major impact on reproductive efficiency in cattle (Ayalon, 1978). Most of the information published deals with infectious, environmental and iatrogenic factors of embryo/fetal loss (Drost and Thatcher, 1994; Vanroose *et al*., 2000) but non-infectious causes probably account for more than 70% of the embryo/fetal death (Vanroose *et al*., 2000). Spontaneous embryo/fetal mortality is defined as the one occurring in an apparently healthy cow/heifer and that is not related to a specific cause. Spontaneous embryo/fetal mortality is the most important component in the reduction of reproductive efficiency (Chapter III). In dairy cows the average calving rate after single insemination is around 50% and in large commercial dairy operations in the US is close to 30-40% (MacMillan *et al*., 1996; Lucy, 2001). In cattle the embryonic period is relatively short (42-45 days; Committee on bovine reproductive nomenclature, (1972) and most of the losses occur during this period (Ayalon, 1978; Kummerfeld *et al*., 1978). Fertilization failure is included in the above percentage. On average in normal healthy cattle the fertilization rate is between 85 and 90% (Boyd *et al*., 1969; Diskin and Sreenan, 1980). Therefore, fertilization deficiency does not account for a high percentage of embryo/fetal mortality. Embryonic mortality is generally suspected when the interval between insemination and return to estrus exceeds the normal 18 to 24 day range (Kummerfeld *et al*., 1978). This interval is affected by the quantity and quality of estrus detection. Most of the cattle embryos are lost well before the critical time of pregnancy recognition (day 16), therefore, the estrous cycle length is not affected (Humblot and Dalla Porta, 1984). A recent study showed that spontaneous late embryo

mortality (from days 30 to 45) is higher than fetal mortality (from days 46 to 60; Chapter III). In a previous observational study, conducted on 551 pregnant dairy cow/heifers in which repeated transrectal ultrasonography was performed during the first four months of pregnancy, two distinct types of clinical, ultasonographic and behavioral embryo/fetal death, were observed (Chapter IV). Little is known about late spontaneous embryo/fetal mortality in cattle. Anecdotal observations in cattle practice allow us to classify spontaneous embryo/fetal mortality in two types based on the uterine characteristics noted by palpation per rectum and ultrasonographic images of the embryo/fetus (JE Romano, unpublished observations). These two types of embryo/fetal mortality were named Type I and Type II. Type I was first noted and characterized by a positive fetal membrane slip by palpation per rectum, presence of embryo/fetus degeneration and a functional corpus luteum. Type II was characterized by absence of an embryo/fetus, absence of positive fetal membrane slip and the presence or absence of a functional corpus luteum. Embryo/fetal mortality Type I was treated with prostaglandin F-2α to induce luteolysis in order to clean the uterus. Females with embryo/fetal mortality Type II were either treated with prostaglandin F-2α if a functional CL was observed or allowed to breed as soon as they showed standing estrus. Therefore, no follow-up at that time on the clinical evolution of each of the types of embryo/fetal mortality was done. The objective of the present study was to reevaluate these previous observations by using palpation per rectum, transrectal ultrasonography, vaginoscopy and sexual behavior. Follow-up was done according to a predetermined schedule of weekly examinations for eight weeks after embryo/fetus death. No treatment was instituted during the period of study. This knowledge could help to understand these types of embryo/fetal mortality, evaluate the reproductive and productive implications and design future preventive measures in order to increase reproductive efficiency.

MATERIALS AND METHODS

This study was conducted in the Dairy Cattle Center of Texas A & M University. Holstein and Jersey cows and heifers were used. Each cow was inseminated after a voluntary waiting period of 55 days. Each heifer was inseminated between 14 and 16 months of age. Lactating cows were housed in concrete dry lots covered free stalls, milked twice daily, and fed a total mixed diet consisting of corn or grain sorghum, soybean meal, alfalfa, and corn silage. Mineral salt and water were offered at libitum. Diets were formulated to meet or exceed NRC requirements (1989). Body condition score was evaluated during the study period using a 1 to 5 scale (Wildman *et al*., 1982). Pregnancy was detected by transrectal ultrasonography between days 29 and 32 after artificial insemination (estrus = day 0). A female was considered pregnant when an embryo with a heart rate of more than 120 beats per minute was detected by transrectal ultrasonography. Each pregnant female was scheduled for further TRUS at days 45, 60, 75 and 120 days of pregnancy. Based in previous experience (Chapter III), once the females were detected pregnant an estrus detection patch was placed on the rump to facilitate the detection of estrus. Late embryonic mortality was defined as embryo death from the time of pregnancy diagnosis (29 to 32) to day 45. Fetal mortality was defined as death or loss of the conceptus during the fetal period ($\geq$ 46 days). Embryonic/fetal mortality was the death or loss of conceptus during the embryonic or fetal period. When a cow/heifer previously diagnosed as pregnant, was diagnosed with a non-viable embryo/fetus, with the embryo/fetus in the process of degeneration or when positive signs of pregnancy were absent by TRUS, she was scheduled for a follow-up in eight weeks. Also, a pregnant female, detected in standing estrus or with an activated estrus detection patch, was scheduled for a complete reproductive evaluation either the same or the next day. Thirty-six animals selected from two previous experiments (Chapters III and IV) were included in this study. Nineteen were Type I which included 10 single pregnancies and 9 with twins. This group included 3 heifers and 16 cows (from 1st lactation to 4th lactation; mean= 2.1). All twin pregnancies were seen in cows: 4 from 1st

lactation, 2 from 2nd lactation, 2 from 3rd lactation and 1 from a 4th lactation. Type II included 16 single pregnancies and 1 twin pregnancy (2nd lactation) and all were cows (from 1st lactation to 5th lactation; mean= 2.1). Each female was evaluated by palpation per rectum, vaginoscopy and transrectal ultrasonography once a week. Sexual behavior was checked once a day by observation for estrus (30-45 minutes) plus utilization of an estrus detection device on the rump (Bovine beacon*). Vaginoscopy was performed using a disposable plastic vaginal speculum. Palpation per rectum was performed to evaluate uterine size, uterine wall thickness and presence/absence of fetal membrane slip. Presence of a positive fetal membrane slip was considered when the allantochorion slipped between the fingers according to the technique described by Zemjanis (1971). All transrectal ultrasonographies were performed by the same operator using an Aloka SSD 500 ultrasound machine equipped with a 5 MHz linear transducer. Time of embryo/fetal death was defined as the mean time between the last time detected alive and the time the embryo/fetus was dead or when the uterus was found clean. Amniotic sac time was considered the mean time between the time of pregnancy detection with an embryo/fetus alive and the last time the amnion was clearly differentiated by transrectal ultrasonography. Allantoic sac time was defined similarly. Degeneration time was defined as the interval from the time pregnancy with a live embryo/fetus was detected by transrectal ultrasonography until the uterus was determined to be completely clean. First estrus time was the interval from the last time the animal was detected pregnant to the first estrus. Estrous cycle duration was the time between the first estrus and the second estrus. Short estrous cycle was considered when the interval was less than 18 days between two estrus period. Pyometra was characterized by a distended uterus containing fluid with uniformly and diffusely dispersed floccules with different degrees of echogenicity, detected by transrectal ultrasonography. In addition, the presence in the ovary, of a functional CL detected by TRUS (>20mm) was required. Ovarian cysts were considered when one or more follicle-like structures more than 25 mm were found in the ovaries. No distinction was made between follicular and luteal types of cysts.

The continuous variables were compared using Student "t" test for independent and for paired samples. The non-continuous variables were compared using Fisher's exact test. A difference was considered significant at a level of $P<0.05$ (Devore and Peck, (1993).

RESULTS

Most of the information is summarized in Table 5. The time that both groups of females were last detected pregnant was 41.6 ± 12.1 days (mean ± SD; range 30-75) for embryo/fetal mortality I and 47.1 ± 22.5 days (32-120) for Type II ($P>0.05$). No change in body condition score was observed between either group before, during or after embryo/fetal death ($P>0.05$). The presence of fetal membrane slip persisted for 16.2 ± 7.9 days (7-28) in Type I and only for 6.0 ± 1.6 days in Type II embryo/fetal mortality (3-7)($P=0.001$). In embryo/fetal mortality Type I fetal membrane slip was positive in 26.3% of cases for 7 days, in 21.0% of cases between 8-14 days, in 31.6% of the cases between 15 and 21 days and in 21.0% of the cases between 22 and 28 days. The amniotic sac and allantoic sac were present for 16.2 ± 1.6 days (7-28) and 17.6 ± 9.5 days (7-35) in Type I and 6.0 ± 1.6 days (3-7) and for 6.0 ± 4.0 days (3-7) in Type II, respectively ($P=0.001$). In embryo/fetal mortality Type I the amniotic sac was evident in 26.3% of the cases for 7 days, in 21.1% of the cases between 8 and 14 days, in 31.6% of the cases for 15 to 21 days and in 21.1% of the cases for 22 to 28 days. In embryo/fetal mortality Type I the allantoic sac was evident in 26.3% of the cases for 7 days, in 21.1 of the cases between 8-14 days, in 21.1% of the cases between 15 and 21 days, in 21.1% of the cases from 22 to 28 days and more than 29 days in 10.5% of the cases. Signs of embryo/fetal degeneration were present for 21.9 ± 10.6 days (7-42) for Type I and 6.0 ± 1.5 days (3-7) for Type II ($P=0.001$). In embryo/fetal mortality Type I the signs of degeneration persisted in 15.8% of cases for 7 days, in 21.1% of the cases between 8-14 days, in 10.5% of the cases from 15 to 21 days, in 26.3% of the cases from 22 to 28 days, in 21.1% of the cases from 29 to 36 days and in 5.3% of the cases for more than 37

days. In twins, embryo/fetal mortality occurred more frequently as Type I (88.9%; 8/9)

Table 5. Comparison of clinical, ultrasonographic and behavioral characteristics between embryo/fetal mortality Type I and II in dairy cattle during the first trimester of pregnancy (mean ± SD).

Characteristics	Type I	Type II	Probability
Number of animals	19	17	
Last time detected as Pregnant (days)	41.6 ± 12.1 (range: 30-75)	47.0 ± 22.5 (32-120)	P= 0.38
Body condition at the Time detected pregnant[1]	2.7 ± 0.2 (2.5-3.0)	2.6 ± 0.3 (2.25-3.25)	P= 0.16
Body condition at the Time detected with Embryo death/open	2.7 ± 0.3 (2.5-3.25)	2.6 ± 0.3 (2.0-3.25)	P= 0.11
Fetal membrane slip (days)	16.2 ± 7.8 (7-28)	6.0 ± 1.6 (3-7)	P= 0.001
Amniotic sac (days)	16.2 ± 7.8 (7-28)	6.0 ± 1.6 (3-7)	P= 0.001
Allantoic sac (days)	17.6 ± 9.5 (7-35)	6.0 ± 1.6 (3-7)	P= 0.001
Signs of degeneration (days)	21.9 ± 10.6 (7-42)	6.0 ± 1.6 (3-7)	P= 0.001
Presence of twins (%)	42.1 (8/19)	5.9 (1/17)	P= 0.015
Presence of singles (%)	57.9 (11/19)	94.1 (16/17)	P= 0.015
Time to 1st estrus (days)	29.5 ± 11.8	5.7 ± 1.9	P= 0.001
Short estrous cycle (%)	26.3 (5/19)	52.9 (9/17)	P= 0.098
Short estrous cycle duration (days)	11.8 ± 4.8	10.0 ± 3.1	P= 0.406
Ovarian cysts (%)	10.5 (2/19)	41.1 (7/17)	P= 0.040
Pyometra (%)	47.4 (9/19)	5.9 (1/17)	P= 0.007

[1]included only lactating animals

than as Type II (11.1%; 1/9) (P=0.001). In single pregnancies, embryo/fetal mortality I was found in 40.7% (11/27) and Type II in 59.3% of the cases (16/27) (P=0.05). The time to the first estrus was 29.5 ± 11.8 days (12-52) and 5.7 ± 1.9 days (3-9) for Type I

and Type II embryo/fetal mortality, respectively (P=0.001). In embryo/fetal mortality Type I the time to estrus was less than 14 days in 10.5% of the cases, between 15 and 21 days in 21.1% of the cases, from 22 to 28 days in 21.1% of the cases, from 29 to 36 days in 26.3% of the cases and more than 37 days in 15.7% of the cases. One cow with embryo/fetal mortality Type I after clearing the uterus was in anestrous throughout the study. The correlation between embryo/fetus age and signs of degeneration was 0.26 (P>0.05). Animals with embryo/fetal mortality Type II showed more ovarian cysts (41.2%, 7/17; P=0.04) and a tendency to have more short estrous cycles (52.9%, 9/17; P=0.098) than females with Type I embryo/fetal mortality (10.5%, 2/19 and 26.3%, 5/19, respectively). Estrous cycle duration was 11.8 ± 4.7 days (5-17) for females presenting Type I and 10.0 ± 3.1 days (6-15) for Type II (P=0.406). The proportion of pyometras in females with embryo/fetal mortality I was higher than in females with Type II embryo/fetal death, 47.4% (9/19) and 5.9 % (1/17), respectively (P=0.007).

DISCUSSION

These results confirm our previous observations that two clinically different types of spontaneous embryo/fetal mortality in cattle exist. Type I is characterized by a positive fetal membrane slip, by a degenerating embryo/fetus, persistence of a functional CL and by a prolonged time to clean the uterus. On the contrary, Type II, is characterized by the absence of fetal membrane slip, clean uterus with no embryo/fetus degeneration and in the majority of cases a non-functional CL. This process was rapid and the uterus was quickly cleaned. In embryo/fetal mortality Type I the embryo/fetus was always dead and finally luteolysis occurred. In embryo/fetal mortality Type II, the inverse seemed to occur; luteolysis was seen first and then the embryo/fetus was expelled.

During the first stages of the embryo/fetal death uterine size did not change in embryo/fetal mortality Type I. At later stages, when degeneration of the conceptus was evident, a reduction in size of the uterine horns which corresponded to the age of the pregnancy was evident. In contrast, in all cases of embryo/fetal mortality Type II

reduction of the uterine size were first noticed. In addition, an increase in uterine tone was characteristic and the lumen of the uterus was always detected empty. In some females free fluid inside the uterus was noted either by palpation per rectum or by ultrasonography. This fluid was the same type seen during the normal periovulatory period in cattle (Kahn, 1994).

In spontaneous embryo/fetal mortality Type I the fetal membrane slip persisted for approximately 2 weeks (range: 7-28 days). The persistence of this positive sign of pregnancy is of great clinical importance because these animals will be diagnosed as pregnant and will either be found open in a future examination or will not calve at the predicted date. The farmer/owner could interpret these findings as the diagnosis being incorrect or that the technique was the cause of the abortion (Momont, 1990). Pregnancy diagnosis by palpation per rectum is standard practice for reproductive and economic purposes especially before 45 days after breeding (Zemjanis, 1971). However, some studies had reported that palpation per rectum at this early stage could be deleterious for the embryo/fetus (Abbitt *et al*., 1978; Paisley *et al*., 1978; Vaillancourt *et al*., 1979; Franco *et al*., 1987). Nevertheless, a recent report showed that spontaneous embryo/fetal mortality was not associated with palpation per rectum when fetal membrane slip technique was used (Chapter III). In the present study, the quality of the fetal membrane slip "feeling" in later stages of the degenerative process was completely different from the normal pregnancy with a live embryo. In these stages, a dry or tacky consistency of the fetal membrane slip was evident. In previous studies in which accidental (Rowson and Dott; 1963) or induced rupture of the amniotic vesicle (Ball and Carroll, 1963, Parmigiani *et al*., 1978) occurred, this procedure was followed by immediate embryo/fetal death. In the manual rupture of the amniotic vesicle the membranes were present by palpation per rectum for 15.9 ± 6.4 days (range: 7-27; Ball and Carroll, 1963). In induced abortion by fetal membrane rupture the uterus was clinically detected as clean by day 16 (range 5-50 days). In another report of induced rupture of the amniotic vesicle between days 41 and 46 of pregnancy a positive fetal membrane slip was evident for 18 days (Kassam *et al*., 1987). Our study agrees with previous reports on induced

embryo/fetal mortality in that it takes a long time to obtain a clean uterus. In contrast, in all cases of embryo/fetal mortality Type II, none of the females presented positive signs of pregnancy at their palpation per rectum at the first scheduled examination. Ultrasonographic evaluation confirmed palpation per rectum findings, presence of a small corpus luteum (<18mm diameter) and the presence of a preovulatory follicle (>10mm).

The lacks of embryo/fetal heart beat as well as absence of pulsation of the umbilical vessels and body movement were the first ultrasonographic signs of embryo/fetal mortality Type I. The embryo/fetus size measured by biparietal diameter, crown-nose length or crown-rump length was reduced compared to the embryo/fetus size of the same age of gestation in control pregnancies (JE Romano, unpublished observations). Later in the process, the allantochorion became detached and floated in the uterine lumen. There was also a reduction in uterine fluid with an increase in echogenicity of allantoic and amniotic fluid. The embryo/fetus was harder to identify as it lost detail and became almost indistinguishable from the rest of the structures. In embryo/fetal mortality Type I signs of conceptus degeneration were observed for around three weeks (range: 7-42). The same results were obtained when embryo/fetal death was induced by amniotic sac rupture (Ball and Carroll, 1963). The interval from application of the abortive procedure to abortion depends on the technique used. When decapitation was used the time was 21.5 days, and 26.6 days when amnion rupture plus fetus crushing was used (Parmigiani *et al*., 1978). In the present study, non significant but positive correlation between age of pregnancy and presence of signs of degeneration was found. This suggests that when the conceptus is older it requires more time for the uterus to clean. This process of degeneration was diagnosed as pyometra by palpation per rectum and transrectal ultrasonography. The proportion of pyometra was higher in embryo/fetal mortality Type I than in Type II. In some cases, a mucopurulent material of varying amount was visible on the vulva, perineum and on the tail. In other cases, this material was expelled from the vagina when the palpation per rectum was performed. At the vaginoscopic examination the secretion was noticed in the speculum or coming out from

the vagina, especially when the cervix was clearly opened. In a study, in which cows had the amnion mechanically ruptured by palpation per rectum, and were later examined post-mortem at day 36, varying amounts of flocculent material in the uterine lumen and presence of a mature CL were found (Kassam *et al*., 1987). Two studies, using mechanical embryo/fetal death in heifers by amniotic vesicle rupture found no passage of membranes or exudates from the genital tract of any of the heifers (Ball and Carroll, 1963; Kassam *et al*., 1987). However, in another study, which used the same abortive procedure a purulent discharge was observed but in only 2 of 21 animals (Parmigiani *et al*., 1978). Differences among studies can be due to the frequency and intensity of observation.

In some cases of embryo/fetal mortality Type II, the vaginoscopic examination revealed presence of mucus and conceptus material at the vaginal fornix or protruding through the cervix. In this last case, vaginal palpation to remove the conceptus was performed. In other opportunities, material which looked-like estrus mucus at a glance was observed hanging from the vulva. In all cases, the material was "clean" with well defined and clear membranes and not muco-purulent material as seen in embryo/fetal mortality II. All of these clinical findings support the concept that the process of embryo/fetal resorption does not occur and that the only process that occurs is "expulsion" as abortion. This means that the dead embryo/fetus is expelled from the uterus rather than destroyed and absorbed inside of the uterus. The old concept of embryo/fetal resorption, possibly, was based in the rapid disappearance of an embryo/fetus during early gestation without observation of conceptus material in the environment of the "pregnant" female in the next reproductive evaluation. The process of embryo/fetal mortality Type II was very quick and therefore, is very easy to overlook. The membranes can easily be misdiagnosed with secretions of a female in estrus as they were translucid and because the animal is around estrus when the process occurs. In all cases of embryo/fetal mortality Type II the embryo/fetus was viable in the last examination, no degeneration of the embryo/fetus was observed as in Type I. One of the most important criteria of this embryo/fetal mortality was the presence of estrus activity

in a female previously diagnosed as pregnant. When prostaglandin F-2α was injected to induce embryo/fetal death in pregnancies of 28 or 42 days, conceptus remnants were detected in the vagina in 45% (9/20) of the heifers (Kastelic and Ginther, 1989). Previous studies reported that many abortions are unobserved particularly during early gestation (Callahan, 1969; Roberts, 1971) and the proportion detected increased with increasing gestational age at the time of fetal loss (Forar *et al*. 1995 and 1996). Only 2.7% (2/75) of embryo/fetal losses were detected before day 68 of the pregnancy (Forar *et al*., 1996). In general, embryos and small fetuses lost in early pregnancy are not found as they could be easily rampled into corral bedding, washed down flush alleys, mixed with manure or carried off by scavengers.

In spontaneous embryo/fetal mortality Type I the time to the first estrus was almost one month after embryo/fetal death was detected (range 12-52). The same results were reported when rupture of fetal membranes was induced in cows around 8.5 weeks of pregnancy (Dawson, 1974). In another study, comparing procedures to induce embryo/fetal death, the interval from amnion rupture to estrus was 38 days and when rupture plus fetus decapitation was used the interval was 32 days (Parmigiani *et al*., 1978). When the activity of the corpus luteum was monitored daily by plasma progesterone after inducing embryo/fetal death by rupture of the amniotic sac, levels were remained high in two cows 35 days after fetal death was induced and in the remaining two cows, regression of the corpus luteum was achieved at 28 and 32 days (Kassam *et al*., 1987). In the present study, 42% of the females with embryo/fetal mortality Type I showed estrus more than 29 days after embryo/fetal death. From all these studies we conclude that after spontaneous or induced embryo/fetal mortality, it takes around one month to restart ovarian activity. Luteal maintenance measured by CL diameter did not require a viable embryo/fetus but required the presence of fluid and membranes in a sterile uterine environment. When Actinomyces pyogenes was infused into the conceptus to induce embryo/fetus death, abortion occurred 144 hours after inoculation (Semambo *et al*., 1991). The inoculation of bacteria changed the conditions of the uterus triggering a very quick process of abortion, probably due to release of

endogenous prostaglandin F-2α. The administration of prostaglandin F-2 α in heifers with conceptus 28-42 days old induced embryo death 2.5 days later (Kastelic & Ginther, 1989). The same results were seen when cloprostenol, was used between days 39-71 (Lindell *et al*., 1980/1981) or when prostaglandin F-2α was injected between days 29 to 52 (Millar, 1974). However, when it was used for older pregnancies (between 102 and 146 days, mean 126.5 days) abortion time was prolonged by one day (3.4 days; Lindell *et al*., 1980/1981)). In all these cases, when the conceptus was seen it was clean, translucid and lacked degenerative changes.

Irregular estrous cycles with shortened luteal phases are known to occur in post-partum cows when resuming estrous activity (Morrow *et al*., 1966; King *et al*., 1976), in post-partum beef cows in which the calf was weaned (Odde *et al*., 1980), following the first ovulation after puberty (Gonzalez-Padilla *et al*., 1975), and after uterine infusion with diluted Lugol solution during the early luteal phase (Seguin, 1980). Preovulatory follicles or premature luteolysis could cause short estrous cycles. Braden *et al*. (1989) suggested that preovulatory follicle characteristics influence the life-span of the sub-sequently formed corpus luteum. Premature luteolysis due to increased serum levels of prostaglandin F-2α and oxytocin can occur in the presence of endometrial oxytocin receptors (Hunter, 1991). The injection of oxytocin during metaestrus suppresses luteal function and shortens estrous cycles in heifers (Armstrong and Hansel, 1959). In goats, the administration of prostaglandin F-2α does not only induce abortion between 24 to 72 hours but causes a variable number of short estrous cycle periods (Bretzlaff *et al*., 1988).

The present study shows that, spontaneous embryo/fetal mortality Type I has several clinical signs similar to embryo/fetal mortality induced by rupture of embryo/fetal membranes or embryo/fetal attrition (Ball and Carroll, 1963; Dawson, 1974; Kassam *et al*., 1987; Kastelic and Ginther, 1989; Parmigiani *et al*., 1978). On the other hand, spontaneous embryo/fetal mortality Type II has similar clinical characteristics to the embryo/fetal mortality induced by the injection of the luteolytic agent, prostaglandin F-2α (Kassam *et al*., 1987; Kastelic and Ginther, 1989).

The question that remains to be answered is: Are these really two types of clinical embryo/fetal death or it is only one process seen differently depending on the methodology used? Further investigations reducing the interval of clinical examinations, including new hormonal profiles and analysis of the conceptus will bring new answers.

CHAPTER VI

PREGNANCY LOSS OF NUCLEAR TRANSFER DERIVED HOLSTEIN EMBRYOS DURING THE FIRST TRIMESTER OF GESTATION

INTRODUCTION

The technique of nuclear transfer (NT) allows the production of embryos, fetuses, and offspring from a range of embryonic, fetal, and adult derived cell types in a variety of different species (Wilmut *et al*., 1997; Baguisi *et al*., 1999; Polejaeva *et al*., 2000; Lin *et al*. 2001; Wakayama *et al*., 1998). The overall efficiency of the technique, however, remains low because only a very limited percentage (0.5-5%) of the transferable embryos result in full-term development (Campbell *et al*., 2001; Yanagimachi, 2002). A high frequency of post-implantation developmental arrest after the transfer of morphologically normal blastocysts has been detected (Stice *et al*., 1996; Hill *et al*., 2000; Campbell *et al*., 2001; Yanagimachi, 2002). In cattle, the embryo/fetal losses were associated with placental abnormalities during the first trimester of pregnancy (Stice *et al*., 1996; Hill *et al*., 2000). Other placental abnormalities such as enlarged placentomes, edematous chorioallantois and enlarged umbilical cord have been observed, especially during the last part of gestation and calving, and these abnormalities are suspected to have compromised fetal health (Hill *et al*., 1999; Wells *et al*., 1999; Heyman *et al*., 2002). Hill *et al*., (2000) recently indicated that mortality in somatic cell nuclear transfer bovine conceptuses during the first trimester of gestation was associated with the absence of cotyledons.

Calves produced from nuclear transfer embryos have been characterized by high birth weight and low survival rate (Bondioli *et al*., 1990; Keefer *et al*., 1994). The cause of these problems has not been identified. Calf size at birth varies within clutches of genetically identical embryos, and the incidence of abnormally large calves approaches 20-30% of the calves born (Bondioli *et al*., 1990; Wilson *et al*., 1995). Studies in mammals suggest that fetuses with abnormal intrauterine growth resulting either in

abnormally large or small newborns, develop more perinatal complications and have greater difficulties in adjusting to extrauterine life in the immediate postpartum period (Holland and Odde, 1992). Birth weight in *in vitro* produced embryos was greater than for calves produced by embryo transfer or artificial inseminated procedures. Nevertheless this accelerated growth did not continue beyond birth (Wilson *et al*., 1995). Most of the information available about large sized NT conceptuses came from late gestation, calving and the neonatal period (Garry *et al*., 1996; Bondioli *et al*., 1990; Wilson *et al*., 1995). Accelerated intrauterine growth of bovine embryos was obtained when cows received exogenous progesterone for four days, starting at 36 hours following mating. The accelerated embryonic growth was evident 14 days post-breeding (Garrett *et al*., 1988). In sheep, accelerated embryonic development was obtained when embryos were transferred into an intermediate host uterus which was chronologically 3 days advanced (Wilmut and Sales, 1981). Closer study of nuclear transfer derived pregnancies during the early period of pregnancy might be able to detect abnormal growth in these conceptuses.

Protein B is a glycoproteic hormone produced by the giant cells of the trophoblast that appears in blood after day 15 post-breeding and consistently at days 24 to 28 after breeding (Sasser *et al*., 1986). This technique showed a maximum sensitivity at days 37/38 (Szenci *et al*., 1998) or later (Humblot *et al*., 1988a). The biological role of this hormone has been suggested (Del Vecchio *et al*., 1995), however, its biological role during pregnancy is unclear. Due to its placental origin, this protein is a useful marker of a viable placenta. Progesterone is a hormone produced by the corpus luteum and is involved in the establishment and maintenance of the uterine characteristics necessary for pregnancy (McDonald *et al*., 1953). The use of luteolytic drugs during the first 5 months of gestation will cause regression of the corpus luteum, resulting in abortion between 2 and 7 days (Barth, 1986; Kastelic and Ginther, 1989). The use of progesterone or progestagens has been effective in maintaining pregnancy after luteolysis, enucleation of the corpus luteum or bilateral ovariectomy (Tanabe, 1966; Zimbelman and Smith, 1966; Lulai *et al*., 1994). The use of protein B as a hormonal marker of placental

hormonal function during early pregnancy could help us to understand how to monitor placental abnormalities in nuclear transfer pregnancies. In addition, progesterone levels, may provide information about their role during the pregnancy loss found in these clone pregnancies.

Previous studies in embryos/fetuses produced by artificial insemination suggested the presence of two types of embryo/fetus death in cattle (Chapter IV and V). One was characterized by the presence of a functional corpus luteum, positive signs of pregnancy by palpation per rectum and signs of embryo/fetus degeneration by transrectal ultrasonography (named: embryo/fetus mortality Type I). The other was characterized by the absence of a functional corpus luteum, negative signs of pregnancy by palpation per rectum and absence of signs of embryo/fetus degeneration by transrectal ultrasonography (name: embryo/fetus mortality Type II). In the first case, the placenta seemed to continue to send information to the endometrium that a conceptus is there, even though the embryo/fetus was detected as dead approximately three weeks earlier. On the contrary, in the second case, the uterus does not recognize the presence of a live embryo/fetus inside, because in general in the last evaluation the heart beat, movement of the fetus or umbilical vessel pulsation was observed in the embryo/fetus. Therefore, if the cause of embryo/fetus death is due to an earlier triggering in the luteolytic mechanism from the uterus the exogenous administration of progesterone or progestagens might be a potentially effective treatment to maintain the progestational characteristics of the uterus necessary to rescue these valuable embryos.

The objectives of the present study were to evaluate the embryo/fetus development, peripheral hormonal profiles of protein B and progesterone and characterize the type of embryo/fetus mortality in nuclear transfer cattle embryos during the first trimester of pregnancy.

MATERIALS AND METHODS

Animals and estrous synchronization

This study was conducted at the Dairy Cattle Center of Texas A & M University. The animals were maintained in a free stall system throughout the investigation period and fed corn silage and high quality hay offered twice a day. Mineral salts and water were offered ad libitum. The animals were synchronized for estrus using controlled internal drug release devices impregnated with progesterone (CIDR; 1.38 g) which were maintained in the vagina for 7 days. All heifers received natural prostaglandin F-2α by intramuscular injection (Lutalyse; 5ml) at the time of withdrawal of the intravaginal device. Estrus was checked three times a day by observation (early in the morning, noon time and late afternoon) after CIDR removal. In addition each female had an estrus detection device on the rump (Bovine Beacon). Time of standing estrus was considered as day 0. After the recipients were embryo transferred a new estrus detection device were placed and they were monitored at least twice a day for signs of illness, sexual behavior and food ingestion.

Nuclear transfer protocol

The fibroblast cell line used during this experiment was derived from one adult lactating Holstein cow (6038; seven-years old). The frozen vial of cells was thawed in 39° C water, and cells were washed with Dulbecco Minimum Essential Medium-F-12 (DMEM/F-12; Gibco BRL, Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories Inc., Logan, UT) for 5 min and then cultured in 25-mm Petri dishes containing DMEM/F-12 + 10% FCS for 2 or 3 days. The fibroblast cells were trypsinized (2% trypsin-EDTA solution for 5 min; Sigma, St Louis, MO) and the dissociated cells were rinsed by centrifuging with DMEM/F-12 + 10% FCS. Some of the cells were used immediately for nuclear transfer and the remaining

cells were cultured in the same medium for later use. Oocytes were purchased from a private company. These were collected from 2-7 mm follicles and were cultured in TCM-199 (Earle's salts, Gibco) supplemented with bovine FSH and LH, sodium pyruvate, 25 μg/mL gentamycin, and 10% FCS. At our laboratory the oocytes were denuded at 20-22 h of maturation by aspirating and expelling repeatedly using a mouth pipette in 0.5 % hyluronidase (Sigma) for 5 min and then washed three times in 20 mM Hepes (Sigma) buffered TCM-199 supplemented with 10% FCS (operation medium). Denuded oocytes were then pretreated for enucleation in a medium supplemented contained 7.5 μg/mL of Cytochalasine and 5μ/mL Hoechst 33342 (Sigma) for 15-20 min. Enucleation was performed by removing the first polar body and a small portion of ooplasm that contained the metaphase II plate and was verified by exposure to ultraviolet light. Micromanipulation was then employed to insert a single donor cell inside the perivitelline space of each enucleated oocyte. The donor cell was juxtaposed to the ooplasm membrane. The oocyte-fibroblast couplets (23-25 h of maturation) were washed twice in fusion medium (0.3 M Mannitol, 0.1mM $CaCl_2$ and 0.1 mM $MgSO_4$ (Sigma) and then moved into the fusion chamber containing fusion medium, and manually aligned. Two electric pulses (2.0 kv/cm, 25 μsec each) were applied. Nuclear transfer units were activated by application of two electric pulses (0.3 kv/cm, 55 μsec each) in fusion medium within 30 min. after application of fusion pulse and then exposed to 10 μg/mL cycloheximide (Sigma) and 5 μg/mL cytochalasin B for 5 h. Fusion was assessed by light microscopy after activation treatment. After activation, NT embryos were washed 3 times in operation medium and then cultured in G1-G2 medium (Vitrolife) in the incubator at 39° C in 5% CO_2 in air. Cleavage rate was evaluated on day 2 after fusion. Morula and blastocyst development was assessed on days 7-8 after fusion. Blastocysts that were spherical, symmetrical with uniform cells size, color and texture and with only a few extruded blastomers were designated as grade 1. These embryos were the only ones used for embryo transfer.

Embryo transfer

Two single fresh embryos with excellent quality were aspirated into a 0.25 ml straw. Immediately after loading, the straw was attached to an embryo transfer gun (Cassou, France) which was wrapped with thermal paper to keep it warm and transported to the farm for transfer. The time from loading to the end of the transfer was less than 3 hours. The transfer were performed on day 7 (estrus= day 0). The transfer gun was inserted into the vagina and was protected with a sanitary sheath up to the cervix in order to avoid contact with the vaginal wall and contamination. Immediately after insertion of the embryo transfer gun two embryos were transferred into the uterus. The site of transfer was located in the central portion of the uterine horn ipsilateral to the ovary with the CL. All embryo transfers were conducted with the same equipment by the same person. To ensure the restraint of the animal at the time of embryo transfer and to facilitate the transfer, each recipient received an intravenous administration of 10 mg of acepromazine acetate two hours before transfer and epidural anesthesia with 4 ml of 2% lidocaine hydrochloride 20-25 minutes before the start of the transfer.

Blood sampling and hormonal analysis

Blood samples were collected from the tail vessels into heparizined and plain tubes and were centrifuged at 1500 X g for 20 minutes after collection. Plasma and serum specimens were stored at -20° C until assayed. Protein B was determined by ELISA assay with a sensitivity of 0.15 ng/ml. The interassay variation was 10.3% and intrassay variation of 7.6%. Progesterone (P_4) was determined by radioimmunoassay with a level of sensitivity of 0.1 ng/ml. Interassay variation was 8.2% and intrassay variation was 4.8%. A progestational stage was considered when the level of progesterone in serum was $\geq$ 1.0 ng/ml.

Embryo/fetus transrectal ultrasonography

All recipients were scheduled for transrectal ultrasonography (TRUS) at day 25 (estrus= day 0) even if they presented sign of estrus after transfer. If an animal was detected as non-pregnant it was re-examined at day 30. All pregnant females were examined every 5 days by TRUS until pregnancy loss and a clean uterus was observed or the fetus was over 90 days old. All transrectal ultrasonographies were performed in the morning, by the same operator, using an Aloka SSD 500 ultrasound machine equipped with a 5 MHz linear transducer. Three measurements: crown-rump length (CRL), bi-parietal diameter (BPD) and crown-nose length (CRL) were evaluated in each embryo/fetus. Time of embryo/fetus death was defined as the mean time between the last time the embryo/fetus was detected viable and the first time detected dead. An embryo/fetus was defined as viable when a heart beat was detected by transrectal ultrasonography. The time required for the uterus to be clean was determined from embryo/fetus death to the time when no detritus or conceptus remnants were seen in the uterus by TRUS. Embryo/fetal death Type I was considered when a fetal membrane slip was determined by palpation per rectum, positive signs of pregnancy in an embryo/fetus death or in process of degeneration were observed plus a CL was present ipsilateral to the uterine horn containing the conceptus when observed by TRUS.

The embryo/fetus measurement and hormonal profiles were compared by variance analysis for repeated measures. The level of progesterone before and after death was compared by “t” Student (Devore and Peck, 1993).

RESULTS

The complete results of pregnancy loss in nuclear transfer derived embryos are shown in Table 6. Three replicates of embryo transfer were performed with 5, 6 and 5 females in the first, second and third replicates, respectively. The total number of pregnancies detected at day 25 was 7 out of 16 transfers (43.8 %). Pregnancy rate for

the first, second and third replicates were 60% (3/5), 16.7% (1/6) and 60% (3/5), respectively. From the seven heifers pregnant, five were detected as having twins and two as singles. The twinning rate was 71.4% (5/7) for all pregnant females. Embryo survival to day 25 for all transferred embryos was 33% (12/32) and for pregnant heifers was 86% (12/14). No differences were detected in CRL, CNL and BPD compared with *in vivo* Holstein embryos produced by artificial insemination (see Figs 5, 6 and 7). No differences were detected between twin clones and single clones at the different stages ($P>0.05$). Only one pregnancy (with single) passed the first trimester of gestation (14.3%; 1/7). Pregnancy loss was 85.7% (6/7). All embryo/fetal deaths were Type I. The time of embryo/fetal death was 50.0 ± 8.8 days. Signs of embryo/fetal degeneration were detected by transrectal ultrasonography for 18.3 ± 6.8 days. No difference in progesterone level was detected between nuclear transferred and *in vivo* derived embryos (see Figure 8). Progesterone level persisted elevated for 16.3 ± 6.7 days after the embryos/fetuses were detected dead (see Figure 9). Protein B remained at pregnancy levels for 20.0 ± 8.2 days after the embryos/fetuses were diagnosed as dead (see Figure 10). However, in 4 out of 6 heifers the level of protein B continued to be detectable

Table 6. Time of pregnancy loss, type of embryo/fetal mortality, hormonal levels of progesterone and protein B in pregnant females with nuclear transfer derived embryos.

Heifer	Pregnancy	Time of Embryo/fetus Death (EFD) (days)	Type of EFD	Time to clean uterus (days)	Time of progesterone persistence (≥ 1.0ng/ml)	Time of positive protein B
139	Twin	47.5	I	20	17.5	22.5*
138	Twin	52.2	I	15	12.5	17.5*
137	Twin	47.5	I	20	22.5	22.5*
130	Singleton	37.5	I	15	12.5	17.5
129	Twin	62.5	I	10	7.5	7.5
1408	Twin	57.5	I	30	25	32.5*
		50.8 ± 8.8	All Type I	18.3 ± 6.8	16.3 ± 6.7	20.0 ± 8.2

*samples with positive results in the last collection

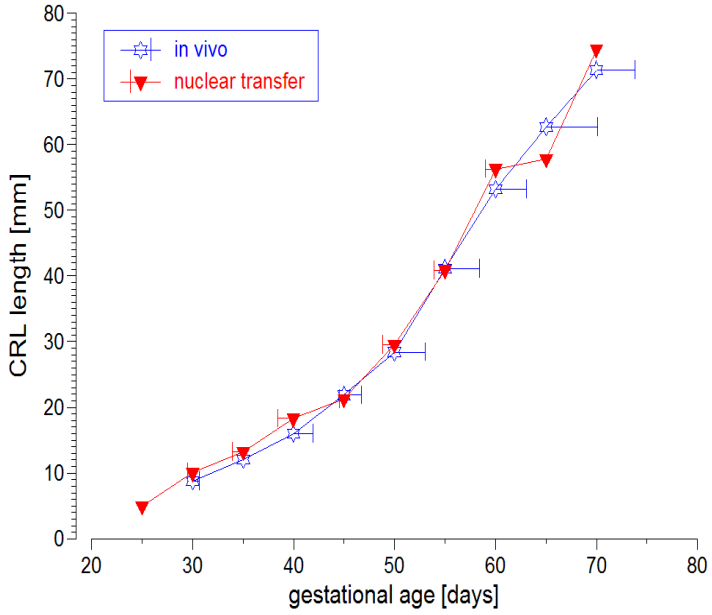

Figure 5. Comparison in crown-rump length (CRL) between nuclear transfer and *in vivo* derived embryos.

(positive results) throughout the collection period. Serum levels of progesterone before embryo/fetus death was 11.3 ± 3.7 ng/ml and was not different from progesterone levels after the detection of embryo/fetus death 12.8 ± 6.9 ng/ml (P>0.05).

DISCUSSION

In the present study, pregnancy rate is in agreement with previous reports using cloned embryos (Wells *et al*., 1999; Hill *et al*., 2000), however, pregnancy rates were lower than results obtained when fresh non cloned embryos were studied (Kruip and den Daas, 1997). The lower pregnancy rate observed in the second replicate (compared to the other replicates using heifers) could be due to higher stress levels in these heifers who were preparing for a ring competition show. The present study unfortunately has a

confounding factor that affects mortality rate: the high twinning rate (5 out of 7 pregnancies carried twins). Twinning increases the risk of spontaneous embryo/fetus death and the proportion of embryo/fetus mortality Type I.

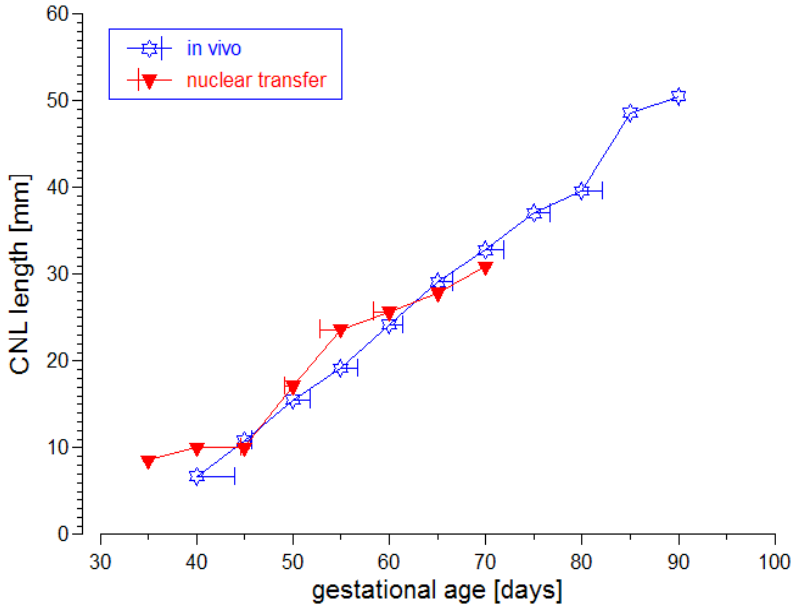

Figure 6. Comparison in crown-nose length (CRL) between nuclear transfer and *in vivo* derived embryos.

Spontaneous twin pregnancies produced by artificial insemination (Chapter III and IV) were previously noted to carry an inherent risk of pregnancy loss almost two and one half times higher than single pregnancies during the first months of pregnancy. Almost 45% of the twin pregnancies were lost during the first four months of pregnancy (Chapter IV). The factors that might contribute to the increased mortality in twins can be competition for nutrients, space or both. In cattle, when twin pregnancies were produced by transferring 2 embryos to each recipient, the number of placentomes at 45-60 days of pregnancy was significatively higher in the case of bilateral transfer compared to the unilateral transfer of both embryos (Rowson *et al.*, 1971). In addition,

in this study, twinning could be responsible for an increased rate of embryo/fetal mortality Type I. A previous study showed that most of the mortality in the spontaneous twin pregnancies was of Type I. Therefore, from the present results a clear conclusion about the type of embryo/fetus mortality in clones cannot be made. In addition, in this study a low number of females were used. The degenerative signs seen in the embryo/fetus were in agreement with a previous study dealing with spontaneous embryo/fetus mortality in *in vivo* produced pregnancies (Chapter IV and V). New studies using more animals and transferring only one high quality embryo would offer a better answer to the questions of the rate and type of embryo/fetus mortality.

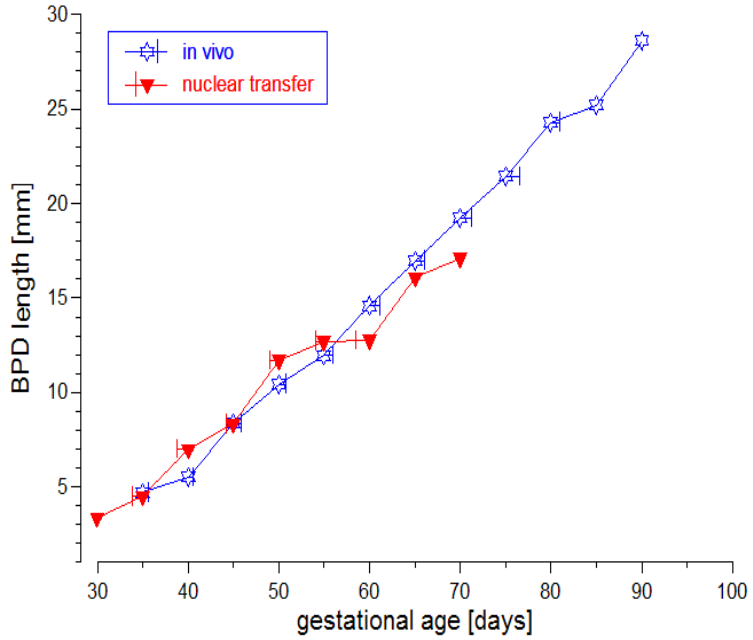

Figure 7. Comparison in bi-parietal diameter (BPD) between nuclear transfer and *in vivo* derived embryos.

The pregnant heifers were considered in good health during and after detection of conceptus death. All females showed good appetite throughout the experimental period. Therefore, a maternal cause of embryo/fetus death can probably be ruled-out. An interesting observation from this study is that all deaths occurred at approximately the same time: middle of the first trimester as previously reported (Wells *et al*., 1998; Stice *et al*., 1996). A previous study reported a pregnancy loss of 82% during the first 3 months of pregnancy (Hill e al., 2000).

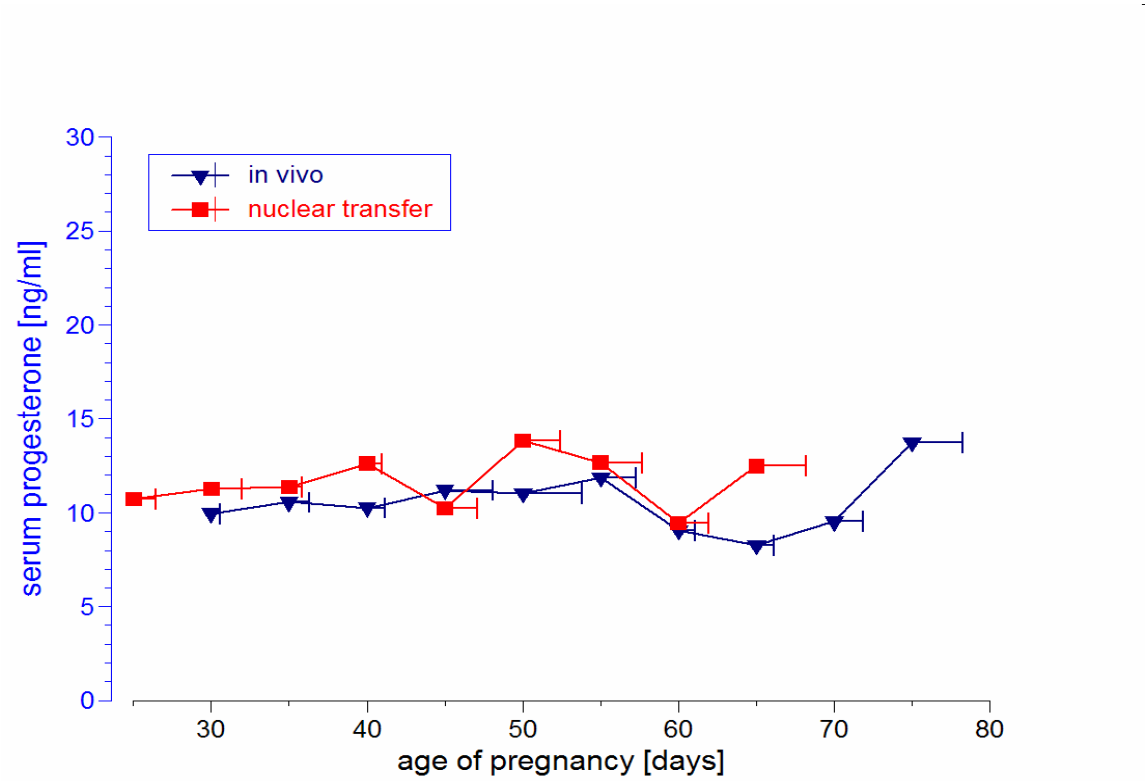

Figure 8. Comparison of progesterone levels between nuclear transfer and *in vivo* derived pregnancies.

No abnormal development, evidenced by the three different measurements used was detected by transrectal ultrasonography in the nuclear transfer embryos. This finding is in agreement with other results in cloned pregnancies that were induced to abort and no abnormality in embryo/fetus was detected (Stice *et al*., 1996; Hill *et al*., 2000).

Another study using females pregnant with Brangus clones slaughtered at day 75 of pregnancy showed no differences in mean fetal length, fetal weight, fetal weight/length index and brain/liver index when compared to conceptuses produced by artificial insemination (De Lille *et al*., 2001). A recent study of nuclear transfer derived Holstein conceptuses at day 50 did not find fetal overgrowth when compared to *in vitro* or *in vivo* produced embryos (Lee *et al*., 2004). Embryo/fetal death appears to be due to an unsuccessful transition from the viteline yolk sac placenta to allanto-chorionic placenta, the time when nutrition of the conceptus passes from the uterine milk to the establishment of definitive allantochorionic placenta. This period corresponds to the transition from the embryo (until day 45) to the early fetal stage (from day 46 on), in which the conceptus nutritional requirements increase dramatically. The daily growth of *in vivo* produced conceptuses increased more than 220 % measured by crown-rump

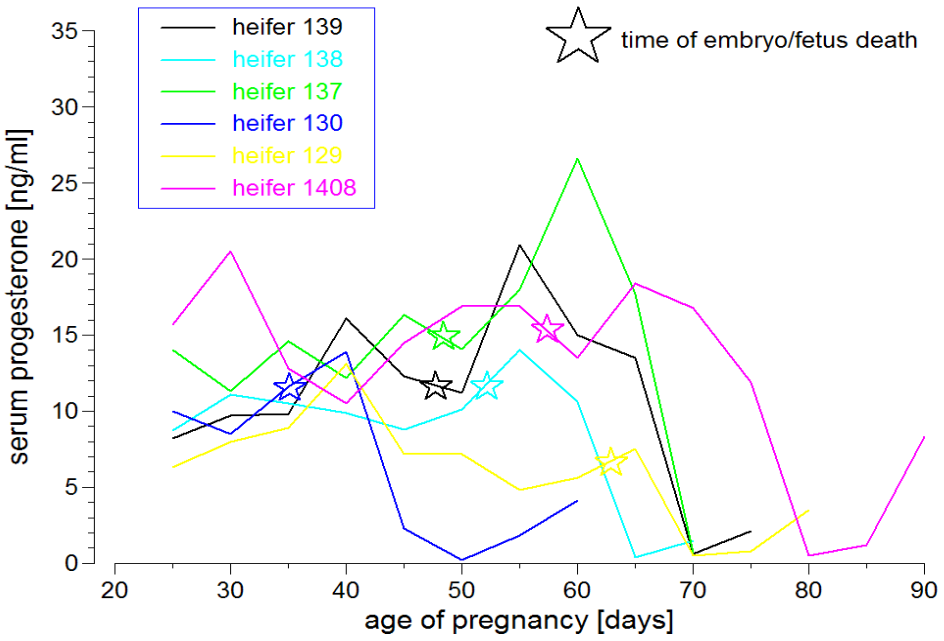

Figure 9. Progesterone level in nuclear transfer derived embryos that were found with embryo/fetus death.

length (CRL) during the early fetal period (CRL growing at 0.98 mm/day from 30 to 50 days changed to 2.15 mm/day from 50 to 70 days; JE Romano, unpublished observations).

From the present study, the causes of embryo/fetus death can not be determined. Placental hormonal marker levels (Protein B levels) in females with dead nuclear transferred embryos were not different from females that carried live nuclear transferred embryos (clone conceptus) beyond 90 days of pregnancy. Levels were also the same as the three control pregnant animals with *in vivo* produced conceptuses. Therefore, the use of protein B as a specific placental marker did not add any information about placental function. The level of protein B persisted positive, at pregnancy level, for three weeks after embryo/fetus death. This could be due to the persistence of production by the giant trophoblast cells or due to the fact that protein B has a long half life (Sasser *et al*., 1986; Sasser and Ruder, 1987). However, it is necessary to point out, that in 4 of 6 animals, the last collection continued to give positive results. This suggests that ther mean values can probably underestimate the true value. The present results do not support the hypothesis that the function of the placenta monitored by their hormonal production was abnormal. However, the placenta is a special temporal organ with multiple functions, of which hormone synthesis is only one. Therefore, we can not rule-out that other functions of this organ were affected (especially when previous studies agreed that macro and microscopic placenta abnormalities were frequent findings in cloned placentas). New placental markers should be evaluated in the future. No differences in peripheral progesterone levels were detected between the females that presented a dead embryo/fetus and the females that carried alive nuclear transferred embryo past the first trimester of gestation nor with the *in vivo* pregnancies produced by artificial insemination. The production of progesterone by the corpus luteum was enough to maintain a progestational stage in all pregnancies, including the ones that were found dead or in the process of degeneration. Therefore, the use of exogenous progesterone as a “potential” treatment for rescue and maintenance of clone pregnancies can not be supported. The progesterone level was maintained in adequate progestational levels for

approximately two weeks after the embryo/fetus was detected dead. In summary, the hormonal production of protein B and progesterone continued to be elevated for some time after detection of embryo/fetus death. The level of these two hormones did not allow us to differentiate the viability status of the conceptus during the observation period. Placentae and corpora lutea continued to recognize the presence of a conceptus without differentiating between alive or dead in the first stages of the degeneration process.

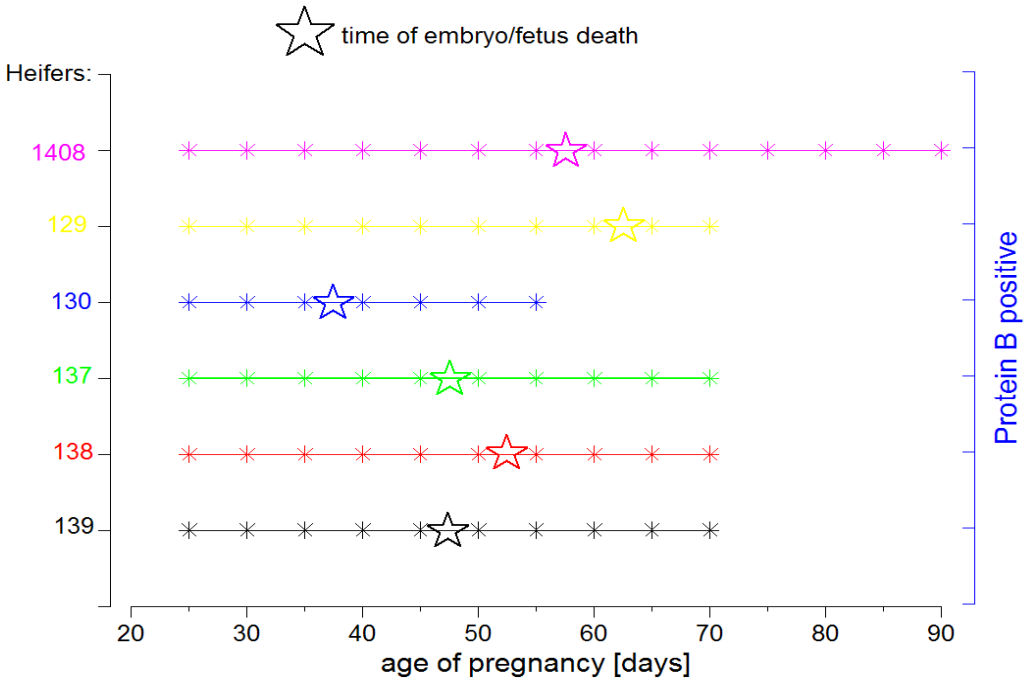

Figure 10. Protein B in nuclear transfer derived embryos that were found with embryo/fetus death.

In general, the most frequent abnormalities detected in nuclear transfer derived conceptuses was abnormal anatomy of placentae evidenced by rudimentary development and vascularization of cotyledons (Hill *et al*., 2000), reduced number of discernable cotyledonary areas (Hill *et al*., 2000), absence of cotyledons with hemorrhage in the

caruncules (Stice *et al*., 1996) or enlarged placentomes and umbilical cords (De Lille *et al*., 2001). One of the causes of the abnormal placenta anatomy could be aberrant allocation of the two different cell lineages observed at the blastocyst stage: the inner cell mass (ICM) and the trophectoderm (TE) cells which will produce the embryo and extraembryonic membranes, respectively. NT blastocysts showed a higher ratio of ICM:total cells compared to *in vitro* and *in vivo* derived embryos (Koo *et al*., 2002). In addition a substantial percentage of embryos produced by nuclear transfer contain chromosomal abnormalities when compared to vivo or *in vitro* produced embryos (Slimane and King, 2002). The rate of abnormalities observed in nuclear transfer derived embryos was higher than those observed in the donor cell line that originated the embryo, suggesting that the nuclear transfer procedure might increase these aberrations (Slimane *et al*., 2003). Another explanation to the higher pregnancy loss observed in nuclear transferred embryos could be an abnormal expression of the major histocompatibility complex (MHC) class I antigens (Hill *et al*., 2002). These antigens are not normally expressed at this stage of pregnancy (Low *et al*., 1990), and their presence might trigger an immunologic reaction that increases the number of lymphocytes inside the endometrium (Hill *et al*., 2002). However, it is important to mention that all these nuclear transfer pregnancies were derived from a single donor cell line. In the same report, when a nuclear transfer pregnancy from a different cell line was examined no MHC expression or T cell infiltration was seen. The process of creating nuclear transfer embryos may modify the expression of MHC class I genes with deleterious results (Ellis, 2004). Nevertheless the abnormal expression of the MHC class I in that single donor cell line could be and inherent genetic abnormality of this cell line. This opens a new area of research to determine if the reprogramming of DNA from the donor cell changes the time of expression of placental antigens.

CHAPTER VII

SUMMARY AND CONCLUSIONS

FIRST STUDY

The sensitivity of TRUS to detect pregnant cows increased gradually from 74.5% at day 24 to 100 % at day 29 ($P<0.01$). Specificity increased from days 24 and 25 and reached a plateau at 96.6% from day 26 on (range: 95.6 to 97.4%; $P<0.01$). The positive predictive value increased in the first two days of evaluation and remained steady after day 26 (range: 89.1 to 93.6%; $P<0.01$). The negative predictive value increased from 88% at day 24 to 100% at day 29 ($P<0.001$). In heifers, the sensitivity increased from 50% at day 21 to 100% at day 26 ($P<0.01$). Specificity increased from 87.5% at day 21 and remaining steady at 94% from day 23 on (range: 87.9 to 96.5%; $P>0.05$). The positive predictive value increased after the first two days of evaluation and reached plateau at 92.8% (range: 86.7 to 96.7%) from day 23 on ($P>0.05$). The negative predictive value was 63.6% at day 21 increasing to 100% at day 26 ($P<0.01$). From this study, it was concluded that the maximum sensitivity and negative predictive value of pregnancy diagnosis were obtained three days earlier in heifers (day 26) than in cows (day 29).

SECOND STUDY

The overall embryo/fetus death was 14.0% (73/520). Embryonic death (10%; 52/520) was significantly higher than fetal death (4.5%; 21/468; $P<0.001$). Embryo/fetus death between palpation per rectum (PAL group: 14.7%; 38/258) and not palpation per rectum group (NPAL group: 13.4%; 35/262) was not significantly different ($P>0.05$). Embryonic death was 9.3% (24/258) for PAL and 10.7% (28/262) for NPAL ($P>0.05$). Fetal death was 5.9% (14/234) for PAL and 3.0% (7/234) for NPAL ($P>0.05$).

Embryo/fetus mortality was higher in twins (25.5%; 12/47) than single pregnancies (12.9%; 61/473; P<0.025). Embryo/fetus mortality for twins was 21.7% (5/23) for PAL and 29.2% (7/24) for NPAL, respectively (P>0.05). It was concluded, that palpation per rectum for early pregnancy diagnosis using the fetal membrane slip technique did not affect embryo/fetus viability.

THIRD STUDY

The overall embryo/fetal mortality in the first four months of pregnancy was 19.2% (106/551). Late embryo mortality (10.9%) was higher than fetal mortality for each period studied (P<0.001). The overall fetal mortality for periods 46 to 60, 61 to 75 and 76 to 120 days was 4.3%, 2.3% and 3.1%, respectively (P>0.05). Embryo/fetal mortality in single pregnancies (16.9%; 84/497) was lower than in twin pregnancies (40.7%; 22/54; P<0.001). In single pregnancies the embryo/fetal mortality for periods 30 to 45, 46 to 60, 61 to 75 and 76 to 120 days was 10.9%, 2.9%, 1.6% and 2.3%, respectively (P<0.001). In twin pregnancies for the same periods it was 11.1%, 16.7%, 10% and 11.1%, respectively (P>0.05). Late embryo mortality between single and twin pregnancies was not different (P>0.05), however, the distribution of mortality in the three fetal periods was different (P<0.01). In single pregnancies, embryo/fetus mortality Type I and II was 41.7% and 58.3%, respectively (P>0.05). In twin pregnancies, embryo/fetus mortality Type I and II was 72.7% and 27.3%, respectively (P<0.05). Embryo/fetus mortality increased with age of the female from 2 to 3 years-old (12.4% to 21.8%; P<0.01) and then remained steady around 22.8% from 3 to 9 years-old. Embryo/fetal mortality in heifers (12.7%) was lower than in cows 21.9% (P<0.025) and no differences between first and fifth lactations were detected (P>0.05).

FOURTH STUDY

The presence of fetal membrane slip persisted for 16.2 ± 7.9 days (7-28) in Type I and only for 6.0 ± 1.6 days in Type II of embryo/fetal mortality (3-7)(P=0.001). The amniotic and allantoic sac were visualized for 16.2 ± 1.6 days (7-28) and 17.6 ± 9.5 days (7-35), respectively for Type I and 6.0 ± 1.6 days (3-7) and 6.0 ± 4.0 days (3-7),

respectively for Type II (P=0.001). Signs of embryo/fetal degeneration were present for 21.9 ± 10.6 days (7-42) for Type I and 6.0 ± 1.5 days (3-7) for Type II , respectively (P=0.001). In twin pregnancies, the embryo/fetal mortality Type I (88.9%; 8/9) was more frequent than Type II (11.1%; 1/9)(P=0.001). In single pregnancies, Type I was seen in 40.7% (11/27) and Type II in 59.3% (16/27), respectively (P=0.05). The time to the first estrus was 29.5 ± 11.8 days (12-52) and 5.7 ± 1.9 days (3-9) for Type I and Type II of embryo/fetal mortality, respectively (P=0.001). The animals with embryo/fetal mortality Type II showed more ovarian cysts (41.2%, 7/17; P=0.04) and a tendency to have shorter estrous cycles (52.9%, 9/17; P=0.098) than females with embryo/fetal mortality Type I (10.5%, 2/19 and 26.3%, 5/19, respectively). Estrous cycle duration was 11.8 ± 4.7 days (5-17) for mortality Type I and 10.0 ± 3.1 days (6-15) for Type II (P=0.406). The proportion of pyometras with embryo/fetal mortality I was higher than in females with Type II embryo/fetal death, 47.4% (9/19) and 5.9 % (1/17), respectively (P=0.007).

FIFTH STUDY

Seven female recipients of nuclear transfer embryos were detected pregnant at day 25, 5 carried twins and 2 with singletons. No differences were detected in CRL, BPD and CNL compared with contemporaneous *in vivo* Holstein embryos/fetuses produced by artificial insemination. No differences were detected in CRL, BPD and CNL between twin and single NT fetuses at the different stages (P>0.05). All embryo/fetus deaths were Type I. The time of embryo/fetus death was at 50.0 ± 8.8 days (mean ± SD). Only one pregnancy (singleton) passed the first trimester of gestation (14.3% of the total pregnant females and 3.1% of the total embryos transferred). Pregnancy loss between pregnancy diagnosis and ninety days was 91.6% from all nuclear transfer embryos at day 25, and 85.7% from all the nuclear transfer pregnancies. Signs of embryo/fetal degeneration were detected by TRUS for 18.3 ± 6.8 days. Progesterone level remained elevated for 16.3 ± 6.7 days after the embryos/fetuses were

detected dead. Protein B remained at pregnancy levels for 20.0 ± 8.2 days after the embryos/fetuses were diagnosed as dead. However, in 4 out of 6 heifers the level of protein B continued to be detectable (positive results) throughout the collection period. Serum levels of progesterone before embryo/fetus death was 11.3 ± 3.7 ng/ml and was not different from progesterone levels after the detection of embryo/fetus death 12.8 ± 6.9 ng/ml ($P>0.05$). It is concluded that no differences were detected among CRL, BPD and CNL measurements between nuclear transfer and AI derived conceptuses. Neither protein B nor progesterone levels obtained before, during and immediately after embryo/fetus death, were able to show abnormal hormonal function from the placenta or corpus luteum, respectively.

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1986-1987	Laboratoire pour le Côntrole des Reproducteurs. Union Nationale des Cooperatives d'Elevage et Insemination Artificielle. Paris. France.
1985	Doctor in Veterinary Medicine College of Veterinary Medicine. University of Uruguay.

PROFESSIONAL EXPERIENCE

2001-2003	Associate Research Scientist. Large Animal Medicine & Surgery. College of Veterinary Medicine. Texas A&M University.
1998-2000	Resident in Food Animal Theriogenology. Large Animal Medicine & Surgery. College of Veterinary Medicine. Texas A&M University.
1988-1995	Assistant Professor in Theriogenology and Animal Physiology. College of Veterinary Medicine. University of Uruguay.
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AWARDS

1999	Clinician of the Year. Student Chapter. College of Veterinary Medicine. Texas A&M University.
1985	Meriex-Interifa-French Government Scholarship. Awarded for best GPA of all Veterinary Graduates in 1985. University of Uruguay.