CAN HEMATOCRIT LEVELS AT ESTRUS IN DAIRY COWS AND SHEEP BE AN INDICATOR FOR PREGNANCY SUCCESS?

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Khaled Elaraby Kelany

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Kimberly Vonnahme
Chair

Dr. Marc Bauer

Dr. Amanda Brooks

Approved:

November 26, 2019
Date

Dr. Marc Bauer
Department Chair

ABSTRACT

Despite the major reproductive advances in the livestock industry, prenatal embryonic loss is still one of the major issues that causes substantial economic loss. While there are many tests available to determine pregnancy soon after maternal recognition of pregnancy, most of the prenatal losses are undetectable because it occurs before then. Based on previous research we hypothesized that increased plasma volume around the time of estrus will increase the survivability of the embryo at early stages of gestation. However, our findings indicate that hematocrit levels are not a consistent measurement in determining successful pregnancies.

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LIST OF ABBREVIATIONS

E2 .....Estradiol 17-β

TAI.....Timed Artificial Insemination

RIA.....Radioimmunoassay

ELISA ..... Immunosorbent assay

PAG.....Pregnancy-associated Glycoprotein

DHEA .....Dehydroepiandrosterone

CHAPTER 1. LITERATURE REVIEW

Prenatal mortality occurs in all mammals. Even though the incidence of prenatal mortality varies between species, there are two approaches to overcome this problem. First, we need to understand why prenatal loss continues to occur despite the natural selection for efficient reproduction. Second, we need to develop practical applications that can increase the survivability of the embryos. Decreasing the incidence of embryonic death in livestock is a very important economic trait, but the biology of this loss may also provide answers to women who suffer from repeated miscarriage and therefore be important for emotional costs as well.

Historically, prenatal development is usually divided into three periods: the period of the ovum, the period of the embryo, and the period of the fetus (Hanly, 1961). In bovine, the time of conceptus attachment, which begins approximately 11 days after fertilization, is defined as the beginning of the embryonic period (Winters et al., 1942). This period is also considered the period of maternal recognition (Hopper, 2014). Interferon-tau, which is released starting on day 12 by the embryonic trophoblast, with peak production on day 15 to 17 is responsible for maternal recognition (Forde and Lonergan, 2017). Interferon-tau is the signal that prevents prostaglandin F2 alpha (PGF2α) release from the endometrium in sheep and cattle (Hopper, 2014). The beginning of the fetal period is marked by the formation of the true placenta, and the completion of the chorioallantoic fusion (Noden and Lahunta, 1985). This process occurs around day 40 to 45 in cattle (Noden and Lahunta, 1985).

According to a Committee on Bovine Reproductive nomenclature, early embryonic mortality occurs between fertilization and day 24 of gestation, late embryonic mortality occurs between days 25 and 45 of gestation, while fetal mortality occurs after day 45 to the end of parturition (Hubbert et al., 1972). For further specificity, some authors choose to classify

embryonic mortality into 3 stages: 1) very early embryonic mortality which occurs between days 0 and 7 after fertilization; 2) early embryonic mortality which occurs between days 7 and 24 after fertilization; and 3) late embryonic mortality which occur between days 24 and 45 after fertilization (Walsh et al., 2011).

Etiological and associated factors

Many factors can lead to prenatal mortality. However, these factors can be divided into three main categories: embryonic factors, maternal environmental factors, and inappropriate relationship between embryo and mother.

Embryonic factors

Abnormal embryos are unlikely to survive due to inherited defects, errors at meiosis or fertilization, or as a result of environmental factors (Wilmut et al., 1986). Embryonic factors can be due to defects in male or female gametes (Hansen et al., 2002). Chromosomal abnormalities play an important role on the male gamete side (Hansen et al., 2002). Inherited factors such as translocation and specific mutations have been linked to prenatal losses in pigs (Akesson and Henricson, 1972) and cattle (Dyrendahl and Gustavsson, 1979). Cytogenetic analysis has been used as a method to detect gross chromosomal abnormalities which was present in 50.0 to 61.5% of spontaneous human abortions in the first and second trimesters (Boue et al., 1975). In another study, 463 out of a 1000 spontaneous abortion in humans had an abnormal chromosome constitution (Hassold et al., 1980). The percentage of chromosomal abnormalities in domestic species is reported to be much less. Frequency of chromosomal abnormalities in early stages of pregnancy (day 2 to 16) in sheep, cattle, and pigs was 14.6%, 7.5% and 10.0% respectively (Wilmut et al., 1986). Furthermore, other studies could not detect abnormalities in sheep

blastocysts in the preimplantation stage (Long and Williams, 1980) and only 1.9% chromosomal anomalies in cattle blastocysts (Hare et al., 1980; Wilmut et al., 1986).

Maternal and managemental factors

Prenatal mortality can occur as a result of an inadequate maternal environment that cannot support a normal pregnancy (Wilmut et al., 1986). The maternal environment can be inadequate due to age, exposure to suboptimal climates, inadequate plan of nutrition, time of insemination, semen quality, and hormonal imbalance.

Age: Maternal age was thought to influence the incidence of embryonic loss (Hanly, 1961). Clinically normal dairy heifers were found to have greater return to estrous rates than multiparous dairy cows in one study (Erb and Holtz, 1958). However, when comparing primiparous heifers, which calved normally and had no retained placental issues to cows that were pregnant two, three, or four times, there was no difference in conception rates (De Kruif, 1978). Furthermore, other studies reported an increasing rate of embryonic loss with increasing parity (Smith and Stevenson, 1995). A recent study reported that the incidence of embryonic loss was not affected by cow parity (Silke et al., 2002). The embryonic loss rate was similar in both cows and heifers (Silke et al., 2002). The pattern of loss, early loss vs late loss, was also the similar for both cows and heifers (Silke et al., 2002).

Perhaps it is not specially age that attributes to the reduction in conception rate, but rather due to the fact that multiparous dairy cows experience additional challenges, such as dystocia leading to uterine infection and improper uterine involution (De Kruif, 1978). We agree with this conclusion.

Climate: Low fertilization rates and high embryonic loss have been associated with increasing ambient temperature and humidity (Stott and Williams, 1962). Cows that were kept in

42 to 45 °C with shade had a 17% reduction in fertility than cows were kept in 32 to 35°C shade. (Stott and Williams, 1962). High humidity increases the effect high temperature. Conception rates decline from 55% to 10% when the temperature-humidity index increased from 70 to 84 (Ingraham et al., 1974). Oocyte competence is important for the survivability of the embryo (Hansen et al., 2002). Improper chromosomal segregation along with other maternal factors such as heat stress and high crude protein diets can lead to oocyte incompetence (Hansen et al., 2002).

The mechanism by which heat stress can cause embryonic loss is thought to be due to its impact on the oocyte, embryonic development, and fetal development (Hansen, 2009). The effect of heat stress on the oocyte can involve changes at the level of the follicle or the secretion of pituitary glands hormones that control the follicular development (Hansen, 2009). In dairy cattle, follicular steroids, estradiol-17β (E2) and progesterone, were reduced by elevated temperature (Bridges et al., 2005). In goats, the follicular response to LH was reduced in response to heat stress (Ozawa et al., 2005). During early embryonic development in cattle, when cows were exposed to heat stress at one day after insemination, the proportion of embryos that developed to a blastocyst on day 8 was reduced (Ealy et al., 1993). However, heat stress had no effect on embryonic development in cows that were exposed to heat stress at day 3, 5, and 7 after insemination (Ealy et al., 1993). Similar results were observed in sheep (Dutt, 1964) and pigs (Tompkins et al., 1967). In the fetal stage, mid and late gestation heat stress can cause redistribution of the blood to the periphery and reduce perfusion of the placental bed (Alexander et al., 1987). This can result in reduced fetal and placental weight (reviewed in Hansen, 2009).

Nutrition: The relationship between fertility and the plane of nutrition has been studied in few experiments. When dairy heifers were fed a low, medium, or high plane of nutrition, 62, 100 and 146% of the Morrison standards, from weaning to the first day of calving, numbers of

service per conception did not differ (Reid et al., 1964). However, heifers that were fed higher plane of nutrition were ready for breeding faster than heifers that were fed lower plane of nutrition. Consequently, conception in heifers that were fed the low plane of nutrition occurred 3 to 4 months later than the other two groups. A low plane of nutrition prior to breeding results in reduced progesterone concentrations and reduced fertilization rate. However, there was no effect on the embryonic death at 8 or 18 days after breeding in beef heifers (Hill et al., 1970). It was established that low plane of nutrition of donor ewes, resulting in lower body weight and body condition, has a negative effect on oocyte quality, which results in lower rates of cleavage and blastocyst formation (Borowczyk et al., 2006). Additionally, the quality of the provided ration can affect the embryonic development.

The effect of nutrition on the ovarian function and embryonic quality is not limited to the quantity of the offered ration but also the quality of this ration. High protein rations can affect the embryonic quality. Excess rumen degradable protein reduced embryonic quality and development when fed to lactating cows (Butler, 1998). Excessive energy levels in the ration of dairy cattle was found to reduce the response to superovulation and alter some gene expression within the developing embryo (Boland et al., 2001).

Time of insemination*:* Despite the fact that an ovum will retain its ability to be fertilized for a few hours after ovulation, embryonic loss increased in dairy cattle if fertilization occurred 6 h after ovulation (Barrett, 1948). Ovulation usually happens 24 to 36 h after the onset of estrus (Pursley et al., 1998). One study reported that the greatest conception rates for artificial insemination (AI) occurred between 4 to 12 h after the onset of estrus which is marked by standing behavior (Dransfield et al., 1998). Similarly, optimized fertilization rates were achieved by AI 12 h after the onset of the estrus (Dalton et al., 2001). When AI occurred 0, 8, 16, 24 or 32

hours after ovulation was synchronized using OvSynch protocol, insemination at 0 h had the lowest pregnancy loss, and the group inseminated at 32 h tended to have the greatest pregnancy loss comparing to the other groups (Pursley et al., 1998).

Semen quality: In a study that used non-return rates, animal not returning to estrus considered successfully fertilized, to estimate embryonic loss, embryonic losses were identical in dairy heifers bred to bulls of different levels of fertility (Kidder et al., 1954). On the contrary, embryonic death was 19.2% in dairy heifers inseminated with semen from low fertility bulls comparing to 10.5% in heifers that were inseminated with semen from high fertility bulls (Bearden et al., 1956). Semen quality, especially sperm head abnormality, can influence the quality of the embryo, suggesting the important role of the male gamete in the early stages of embryogenesis (Parinaud et al., 1993).

Hormonal imbalance: Estradiol-17β concentrations were greater in cows that conceived particularly 12 h before estrus and for 8 days afterwards (Erb et al., 1976). In the same study, progesterone concentration was greater in cows that conceived at both 48 to 32 h before the LH surge and 6 days after ovulation compared to cows that failed to conceive (Erb et al., 1976). The authors suggested that early failure of pregnancy maybe associated with suboptimal progesterone levels prior to LH surge, E2 and progesterone during certain periods after ovulation (Erb et al., 1976).

The preovulatory E2 secretion and postovulatory progesterone secretion have been shown to affect pregnancy establishment and maintenance (Pohler et al., 2012). Preovulatory follicle maturity affects the follicular microenvironment which can impact the preovulatory secretion of E2 and postovulatory secretion of progesterone along with the oocyte competence (Pohler et al., 2012).

Many researchers investigated the effect of progesterone on fertility in cows over the years. A meta-analysis was performed using data from 53 publications involving control (n = 9905) and progesterone-treated (n = 9135) cows (Yan et al., 2016). Providing progesterone was found to increase the pregnancy rate odds ratio (Yan et al., 2016) but only in cows that were observed in estrus, and not in cows that were treated with progesterone following synchronization of estrus or, regardless if they were observed in estrus (Yan et al., 2016). It was suggested that cows mated to a natural estrus might suffer from a delayed postovulatory progesterone rise leading to asynchrony between the embryo and the uterus, while a synchronized estrus can avoid this issue through the administration of progesterone (Yan et al., 2016). Thus, additional progesterone was only beneficial in cows inseminated following natural estrus. Additionally, progesterone treatment was not beneficial when provided at day 3 to 7 after insemination and to cows with record of good reproductive performance, defined as exhibited a normal estrous cycle, no reproductive disease, and a proper calving interval (Yan et al., 2016).

Other authors think that progesterone can improve fertility rate in cattle (Anim and Hansen, 2011). However, efforts to improve fertility rate using progesterone have mixed success due to insufficient dosage or delayed administration of progesterone (Anim and Hansen, 2011).

In conclusion, increased progesterone levels can result in increase in fertility rate. However, efforts to increase fertility through administering progesterone are not necessarily successful due to the various reasons that were mentioned before.

Uterine environment: Embryonic development in the preimplantation stage is dependent on a substance produced by the endometrial glands called histotroph (Lonergan et al., 2016). Conceptus elongation does not occur in vitro, or when the absence of uterine glands was induced in vivo because of the absence of histotroph (Lonergan et al., 2016).

Progesterone from the corpus luteum has an vital role in inducing histotroph production which contain growth factors that stimulate growth acceleration in the embryo (Barnes, 2000). Additionally, maternal progesterone regulates conceptus growth and development (Lonergan et al., 2016).

Conceptus growth and development is extremely important, as the more space the conceptus occupies within the uterus allows for greater delivery of interferon τ to the uterine epithelium , preventing the production and release of PGF2α from the endometrium (Spencer et al., 2008). Failure of pregnancy recognition is thought to be responsible for up to 25% of failure of conception in dairy cows (Sreenan and Diskin, 1983).

Additionally, uterine inflammatory diseases, that can be caused by bacterial or viral infection, can negatively impact oocyte fertilization, development of the zygote to the morula stage and impair conceptus elongation and consequently the secretion of interferon tau (Lonergan et al., 2016).

Uterine asynchrony

Embryos might not survive because they are not at the correct stage of development for the particular uterine environment despite the fact that both embryo and maternal environment are normal (Wilmut et al., 1986). Embryo transfer that was performed with animals that were not in estrus at the same time was not successful, suggesting that embryo development during early pregnancy depends on a sequence of changes in uterine secretion (Noyes and Dickmann, 1960; Wilmut and Sales, 1981). Similar results were obtained from different studies that were carried out in sows and sheep to confirm the effect of asynchronous environment on embryo survivability (Wilmut et al., 1985; Galvin et al., 1993). Additionally, it was established that 20% to 30% of viable embryos are lost by d 18 due to uterine asynchrony (Ford et al., 2002).

Interestingly, ovine embryos increased their cell division when introduced to an advanced uterine environment, and slow their cell division when introduced to a delayed uterine environment and as a result, pregnancy proceeded normally but the gestation length varied (Pope, 1988). However, if there is more than a 3 day difference between the donor and recipient in embryo transfer, it is unlikely that pregnancy will occur (Ashworth, 1992).

As mentioned before, progesterone can affect the uterine environment directly through the production of uterine histotroph and indirectly through the elongation of the conceptus. It was suggested that uterine-embryo asynchrony, specifically in embryo transfer, can be overcome by appropriate progesterone administration (Ashworth, 1992). However, as mentioned before progesterone administration has limited success. It was suggested that the administration of human chorionic gonadotropin (hCG) can increase progesterone levels and had more success in increasing fertility rates (Anim and Hansen, 2011).

The incidence of prenatal death

Prenatal loss incidence in humans

The percentage of successful pregnancies for women having frequent intercourse without using contraception is only 18 to 28% of menstrual cycles (Short, 1985). The ability to detect hCG, a hormone produced by the placenta after implantation, has helped to detect pregnancy in humans as early as 6 to 18 days after ovulation. The losses that occurred before day 18 have not been recognized (Wilmut et al., 1986). The production of hCG starts as early as the 6-8 cell stage, 2-3 days after fertilization. However, it becomes detectable in mother's blood and urine between 6-14 days after fertilization (Nepomnaschy et al., 2008). A study was conducted to measure the incidence of pregnancy loss by monitoring hCG levels during 207 cycles of 82 women who wished to conceive found that 61.9% of conceptuses were lost prior to 12 weeks.

Most of these losses (91.7%) occurred subclinically without the knowledge of the mothers (Edmonds et al., 1982).

Prenatal loss incidence in ewes

Some studies estimated the embryonic loss in ewes as 28.6% (Hulet et al., 1956). Most of the embryonic loss occurred in the first 18 days after fertilization (20.4%), with the remaining loss occurred throughout the rest of gestation. The embryonic loss was estimated by comparing 18-day non-return rates to the fertilization rates in ewes bred to the same stud rams (Hulet et al., 1956).

Embryonic loss was found to be 43.3% of the potential offspring (Dixon et al., 2007). Early embryonic losses, before d 25 of pregnancy, accounted for 28% of the pregnancy loss, whereas late embryonic and fetal losses account for 15.3% (Dixon et al., 2007). Dixon`s estimation of pregnancy loss is more reliable considering that ultrasonography was used to determine the embryonic loss after d 25 and early embryonic losses were estimated through fetal number present on day 25 minus the number of corpus luteum present on the ovary (Dixon et al., 2007). Overestimation of the percentage of embryonic loss is possible because fertilization failure can be mistaken for early embryonic loss. However, it should be noted that the ewe has ~91% of all ova being fertilized, therefore, this potential overestimation of embryonic loss can be diminished (Bolet, 1986).

Prenatal loss incidence in cattle

The increase in the interval between service and return to estrus beyond the average range 17 to 25 days is thought to be due to early embryonic loss. In a study that includes a total of 9,302 fertilizations, 1,915 embryos (20.6%), were lost (Erb and Holtz, 1958). In another study, 90% of pre-insemination estrous cycles were of normal length, while only 43.5% of post-

insemination cycles were of normal length (Boyd, 1973). This indicates that the extended inter-estrous interval is due to early embryonic loss in inseminated animals, and these losses happen at early stages of pregnancy.

Using increased interval between insemination and returning to estrus as a method of estimating the incidence of embryonic loss faces three major objections. First, progesterone concentrations in blood indicated that up to 20% of the cows presented for insemination had elevated progesterone levels, meaning that they were not actually in estrus (Appleyard and Cook, 1976). Secondly, uterine infection, which can occur after insemination, is associated with a persistent corpus luteum which can delay the cow's return to estrus (Woody and Ginther, 1968). Third, the major portion of embryonic loss can occur before day 15 after insemination. Therefore, the embryo dies too early to prevent the uterus from secreting the PGF2α needed to regress the corpus luteum. Therefore, these cows will return to estrus at the same interval as non-inseminated cows (Ayalon, 1978).

In cattle, studies were conducted with animals with no infertility history and animals with infertility history, i.e., "repeat breeders." Animals with clinically obvious reasons of infertility, such as uterine infection, cystic ovaries, etc., were excluded in most studies (Hanly, 1961). Hawk and his coworkers were able to recover normal embryos from 58% of repeat breeder cows 16 days after the first day of heat. In another group where conceptuses were collected 34 days after estrus, normal embryos were recovered from 28% of the cows. The estimate of embryonic death from 16 to 34 days was 51.7% (Hawk et al., 1955). Since their estimation was similar to other studies that were conducted in the period of fertilization to 34 days, they concluded most embryonic death occurred between 16 and 34 days (Hanly, 1961).

Prenatal loss in dairy cattle

In the 1980s, fertilization rates, or the percentage of cows fertilized of the total cows being inseminated, in Holstein-Friesian dairy cows ranged from 90 to 100%. However, as average milk production increased over the years, fertility has decreased to 83% (Sreenan and Diskin, 1986; Sartori et al., 2009). In contrast, fertilization rates in heifers have remained above 90% (Diskin and Morris, 2008; Sartori et al., 2009). Despite this high fertilization rate in heifers, Holstein pregnancy rates (i.e., the percentage of cows eligible to become pregnant in a 21 day period) in cows are only 39% (Badinga et al., 1985; Diskin and Morris, 2008). This variation between fertilization rate and pregnancy rate is mainly due to the embryonic death. The estimate of both fertilization failure and early embryonic mortality range between 20 and 45%, while estimates for both late embryonic and fetal mortality ranged between 8 and 17.5% (Humblot, 2001). These numbers indicate that most of embryonic mortality occurs in the first 3 weeks of pregnancy; however, there is a controversy about the timing of this loss. While some studies report most early embryonic loss occurs within the first week after fertilization, others think it occurs mostly within the second week after fertilization (Sartori et al., 2002; Diskin and Sreenan, 1980).

We described some etiological factors that induce embryonic death. Here, we are trying to associate theses causes with the specific stage of embryonic development.

Very early embryonic mortality (days 0-7): The three main reasons that lead to embryonic mortality within the first 7 days of pregnancy are 1) low quality of the oocyte, 2) inadequacy of the oviduct and uterine environment, and 3) decreased plasma progesterone levels at very early stages of pregnancy (Walsh et al., 2011).

Oocyte quality has been linked to genetic merits and physiological status (Walsh et al., 2011). Regardless of actual milk production, cows with high genetic merit for milk production produce lesser quality oocytes than cows that have medium genetic merits for milk production (Snijders et al., 2000).

Physiological status of the cow (lactating or non-lactating) was reported to influence embryo quality. Embryos collected from non-lactating Holstein heifers and beef cows on day 7 after estrus were of greater quality than embryos collected from lactating Holstein cows on day 7 (Leroy et al., 2005). Similar results were reported for embryos collected at day 5 of gestation from Holstein heifer vs lactating Holstein cows (Sartori et al., 2009).

The adequacy of the oviduct and uterine environment is very important for the survival of the embryo. The very early embryo remains in the oviduct for 4 to 5 days after ovulation before entering the uterus. The oviduct provides nutrients and local growth factors to the conceptus (Robinson et al., 2008). These oviductal nutrients and growth factors can be manipulated by maternal nutrition (McGuire et al., 1992; Pushpakumara et al., 2002). Disrupting the mechanism of the oviductal muscular and ciliary movement may present the embryo to the uterus and the wrong stage of development leading to very early embryonic mortality (Wiebold, 1988).

The uterine environment for lactating dairy cows may also be suboptimal for supporting early embryonic development comparing to the uterine environment of heifers. In a recent study, using endoscopic transfer technique, 1,800 in vitro-produced embryos were transferred into the oviducts of Holstein heifers and postpartum lactating Holstein cows. The recovery rates were greater in heifers (79%) than cows (57%). Furthermore, 34% of the recovered embryos from the heifers developed to the blastocyst stage compared to only 18% in cows (Rizos et al., 2010).

Lower circulating progesterone levels in lactating cows were thought to be the reason behind the lesser ability of the uterine environment to support the embryo development (Rizos et al., 2010).

Early embryonic mortality (days 7 to 24): As the embryo descends to the uterus between day 5 to 7, an optimal uterine environment becomes essential to support embryonic development. Reduced progesterone concentrations and therefore reduced histotrophic growth factors can create a suboptimal uterine environment that is necessary for embryonic development (Rizos et al., 2002; Leroy et al., 2008). Uterine infection can also affect the uterine function and lead to early embryonic death (Sheldon et al., 2006).

Late embryonic and fetal stage mortality (days 24 to 285): The incidence of pregnancy losses during the late embryonic and early fetal stage vary based upon management systems. Embryonic losses in cows on pasture based systems is approximately 7% in lactating cows and 6% in heifers, with 48% of this losses occurring between days 24 and 42 of lactation (Silke et al., 2002). The embryonic loss rate in an intensively managed dairy (i.e., milk yield between 11,000 and 12,000 kg of milk per lactation) was 20% with most losses occurring between 28 and 98 days of lactation (Vasconcelos et al., 1997).

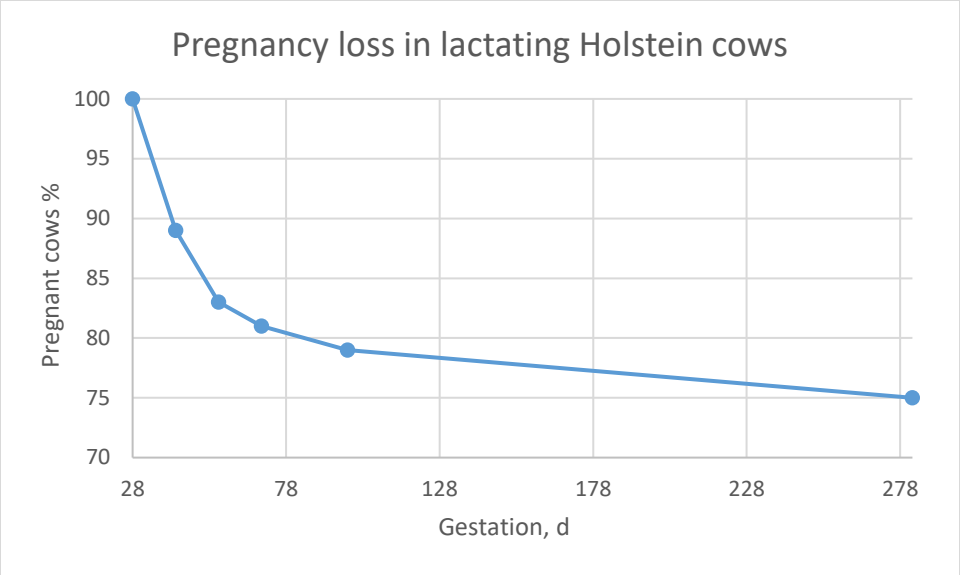

Figure 1. Pregnancy loss in lactating Holstein cows assessed using transrectal ultrasonography from 28 days after AI to calving. Data redrawn from Vasconcelos et al., 1997.

Besides the before mentioned factors that contribute to embryonic loss (age, nutrition, environment and climate), a wide range of pathogens including bacteria, viruses, fungus, and protozoa have been associated with abortion and infertility in cattle. This subject was reviewed by Givens and Marley, 2008 and will not be further discussed in this literature review.

Table 1. Timing and potential causes for prenatal loss in dairy cattle.

	Embryonic loss			Fetal loss
	Very early	Early	Late	
Timing (d)	0 - 7	7 – 24	24 - 45	45 – 282
Incidence	20 - 45%		8 - 17.5%	
Causes	Low quality oocyte Inadequate oviduct environment Low plasma P4	Inadequate oviduct environment Low plasma P4 Uterine infection	Age Nutrition Climate Pathogens	Age Nutrition Climate Pathogens

Embryonic loss effect on dairy farm economy

Reproductive efficiency is one of the major factors affecting dairy farm profitability (Groenendaal et al., 2004; Meadows et al., 2005). Many studies recommended 12 to 13 months as an ideal calving interval (Holmann et al., 1984; Schmidt, 1989; Meadows et al., 2005).

Several economic models suggest different costs for additional days open after the ideal calving interval. Some suggest a modest cost for days open of $0.04 to $0.23 per day for each day beyond 13 months (Holmann et al., 1984). In a later study which account for culling for reproductive failure, the cost of additional days open was $0.96, $2.72, $4.56 for a 1 day increase in adjusted calving interval, at 12, 13, and 14 months, respectively (Plaizier et al., 1997). Clearly these estimates are greater than the previous estimates, but Plaizier and colleagues (1997) take into account the joint effect of culling and the increasing cost of days open when reproductive efficiency declines.

The cost of increasing days open does not come solely from the increase in feed cost, but also from failure in producing enough replacement heifers. According to the model developed by Meadows et al. (2005), the number of mature heifers produced in the cow's life decreases to less than one as days open increase from 145 to 150. When replacement heifers are not produced, farmers are forced to purchased new replacement heifers that are ready to calve to maintain their desirable milk production which explain the economic loss (Meadows et al., 2005).

Methods for early pregnancy detection

As previously mentioned fertilization rate in dairy cattle is 83% and according to older reports can go up to 95 to 100% (Sreenan and Diskin, 1986; Sartori et al., 2009). Early embryonic loss reduces pregnancy rates to 39% (Diskin and Morris, 2008). To diminish this effect and avoid an extended calving interval, early pregnancy detection is very important to identify non-pregnant cows and rebreed them as soon as possible.

According to Fricke and coworkers (2016), the ideal pregnancy test for dairy cows should fulfill these criteria: 1) high sensitivity (i.e., correct identification of pregnant animals); 2) high specificity (i.e., correctly identify non-pregnant animals); 3) inexpensive; 4) simple to conduct

under farm condition; 5) have the ability to determine pregnancy status at the time the test is performed; and 6) have the ability to determine pregnancy status non-invasively.

Non-pregnant cows often require a hormonal intervention to bring them back to estrus for rebreeding. Thus, identifying non-pregnant females correctly is very important, failure in achieving this (false negative) will increase the rate of iatrogenic pregnancy loss, a loss caused by the medical examination, when $PGF_{2\alpha}$ is administered to achieve estrous synchronization (Fricke et al., 2016). However, identifying pregnant cows is more important from economic perspective (Giordano et al., 2013).

The first indication of pregnancy is if the cow is not returning to estrus from 18 to 23 days. Although, this indicator cannot be considered as a method for pregnancy diagnosis for various reasons (Fricke et al., 2016). First, estrous detection efficacy is less than 50% in most U.S. dairies (Senger, 1994). Second, there is a great individual variation is estrous cycle length (Remnant et al., 2015). Finally, early embryonic mortality will increase the interval from insemination to returning to estrus (Ricci et al., 2014). Pregnancy detection can be categorized into direct and indirect methods. Direct methods will involve direct detection of the conceptus and/or the associated fluids, whereas indirect methods will use qualitative or quantitative measures of hormones or conceptus-specific substances in maternal body fluids as an indirect indicator of a viable pregnancy (Fricke et al., 2016).

Direct methods for pregnancy detection

Transrectal palpation: This method is one of the popular methods for pregnancy diagnosis and it was first described in the 1800s (Cowie, 1948). This method involving palpation of the amniotic vesicle through the rectal wall (Wisnicky and Casida, 1948). Because of the risk for manual rupture of the amniotic vesicle (Ball and Carroll, 1963), the relationship between

transrectal palpation and early embryonic mortality has been investigated. While some studies report an increase in the risk of iatrogenic pregnancy loss (Abbitt et al., 1978; Paisley et al., 1978), other studies report that cows submitted to transrectal palpation had limited to no risk of pregnancy loss (Thompson et al., 1994).

Transrectal ultrasonography: This method displaces transrectal palpation as the direct method of choice for pregnancy diagnosis (Caraviello et al., 2006). Various reasons encourage veterinarians to make the transition from transrectal palpation to transrectal ultrasonography. For example, transrectal ultrasonography is a less invasive technique than transrectal palpation because it involve less manipulation of the amniotic vesicle, and it has not been implicated as a cause for early embryonic loss (Mee et al., 1994). Additionally, transrectal ultrasonography can be used to evaluate ovarian structures, aiding in the determination of hormonal intervention. Furthermore, it can identify cows carrying twins and determine fetal sex (Mee et al., 1994).

Many studies reported that transrectal ultrasonography can be successfully performed around day 21 (Curran et al., 1986; Scully et al., 2014). Despite the fact that these studies were conducted under controlled experimental conditions using high-quality scanner and transducer, many practitioners start pushing the lower limit for early pregnancy diagnosis to less than 30 days after insemination (Fricke et al., 2016).

Use of transrectal ultrasonography before day 30 after insemination was evaluated in a field trial using approximately 2,000 cows from commercial dairies. The authors concluded the accuracy of transrectal ultrasonography dramatically increased after day 30 of insemination, when an embryo with a heartbeat can be rapidly and reliably detected (Fricke et al., 2016).

Direct methods for pregnancy diagnosis are very popular in the modern dairy farm. However, they fail to meet all the criteria for ideal pregnancy test. While transrectal palpation

can be inexpensive and easy to perform, it can be less accurate and invasive at early stages of pregnancy. On the other hand, transrectal ultrasonography is less invasive and more accurate; however, it is an expensive method and required trained personnel. Additionally, both methods are performed after day 30 which make both of limited use to diagnose early embryonic loss.

Indirect methods for pregnancy diagnosis

***Progesterone*:** Progesterone is produced from corpus luteum during the estrous cycle through the pregnancy, and in later stages of gestation, from the placenta. Quantification of progesterone in milk or blood is done in a laboratory using radioimmunoassay or enzyme-linked immunosorbent assay methods. There are available on-farm tests designed to determine relative progesterone concentration (i.e., high or low) rather than obtaining a precise concentrations (Nebel, 1988). Cows with low progesterone levels 18 to 24 days after AI would be classified as not pregnant, whereas, cows with high progesterone for the same period would be classified as pregnant (Fricke et al,. 2016).

Although progesterone concentrations can be very accurate in identifying non-pregnant cow at early stages of pregnancy (i.e., 18 to 24 days), the pregnant outcome accuracy is poor. The poor accuracy is attributed to early embryonic loss which can cause extended luteal phase (Ricci et al., 2014).

Pregnancy associated factors: Proteins and other factors produced by the fetus and placenta in early pregnancy stages are obvious candidates for an early pregnancy test (Fricke et al., 2016). Human chorionic gonadotropin is required for luteal support during early pregnancy and is used for pregnancy detection in human (Cole, 2009). Unfortunately, ruminant conceptuses do not produce a chorionic gonadotropin. Different pregnancy-associated compounds have been used to determine pregnancy status. Pregnancy-associated factors such as early conceptus factor

has been discovered but have not proven accurate for dairy cows (Cordoba et al., 2001). Interferon-tau is being secreted by embryonic trophoblast cells and is greatest at day 15 and 17 and observed up to day 28 of pregnancy (Hopper, 2014). Measuring interferon-stimulated genes expression in circulating blood can detect viable conceptus between 15 to 22 days (Stevenson et al., 2007; Green et al., 2010). However, these methods have not yet been commercialized (Fricke et al., 2016).

***Pregnancy-associated Glycoprotein (PAG)*:** Bovine PAGs have been immunologically localized to trophoblast binucleate cells present in the fetal cotyledonary villi and caruncular epithelium (Zoli et al., 1992). Granules that contain PAGs enter the maternal circulation during the immigration of binucleate cells from the trophectoderm to the uterine epithelium (Wooding, 1992). Detection of PAGs in the maternal circulation can start from day 15 to 35 of gestation (Giordano et al., 2012). However, using of PAGs as a pregnancy test is not applicable until day 26 to 30 after AI because of the variation in PAG level between cows (Humblot, 2001). Based on plasma and milk PAG profiles, the optimal time for conducting a first pregnancy diagnosis will be around 32 days after AI, after that PAG levels will drop until day 74 when a rebound in the PAG can be detected (Fricke et al., 2016). However, because of pregnancy loss and the long half-life of PAGs, a second test should take place 74 days after AI when plasma and milk PAG levels have rebounded (Fricke et al., 2016). When PAG pregnancy test results are compared to transrectal ultrasonography, accuracy was 92% for plasma PAG test and 89% for milk PAG test 32 days after insemination (Fricke et al., 2016).

In conclusion, the indirect method for pregnancy diagnosis can be performed earlier than the direct methods with acceptable levels of accuracy. However, both direct and indirect methods are not able to predict future embryonic loss and, therefore, most producers will have to perform

another pregnancy test. Thus, the need to develop a method that can identify the animals that can experience embryonic loss still exist.

Table 2. A summary of differences between pregnancy diagnosis methods.

	Pregnancy diagnosis method				
	Direct		Indirect		
	Palpation	Ultrasonography	Progesterone	Preg. Proteins	PAG
Days post fertilization	45	30	18-24	15-22	26-30
Availability to producers	Always	Sometimes	Sometimes	-	Sometimes
On-farm results	Instant	Instant	Delayed	No	Delayed
Need to recheck	Yes	Yes	Yes	Yes	Yes

Ovarian steroids and plasma volume

Ovarian steroids, estrogen and progesterone, play an important role in preparing the uterus for successful implantation and support embryonic growth and placentation (Johnson et al., 1997a; Johnson et al., 1997b; Koos, 2011). Estrogens, namely E2, and progesterone, in various combinations and durations of exposure, increase endometrial cell proliferation, vascular growth, and microvascular permeability (Reynolds, 1949; Reynolds et al., 1998;Koos, 2011). Ovarian steroids have effects on blood circulation involving mechanisms that control blood flow (Sarrel, 1990). Recent studies in beef cattle reported the increase in embryonic survival rates before day 7 of pregnancy was dependent on the increase in the ovulatory follicle size and, consequently, the increase in E2 production (Atkins et al., 2013). In a different study, supplementation of estradiol cypionate, a synthetic estrogen, during the preovulatory period, increased pregnancy rates in beef cows (Jinks et al., 2013).

We suggest that the relationship between elevated E2 and increased pregnancy rates can be explained by the effect of E2 on blood volume. Circulating renin and angiotensin levels are

regulated discordantly by estrogen status (Schunkert et al., 1997). The renin-angiotensin system is involved in the hemostasis of peripheral vascular resistance, volume, and electrolyte composition of body fluids (Peach, 1977). Therefore, changes in blood circulation during pregnancy seem to be controlled by E2.

In the non-pregnant state, adequate blood volume is necessary for normal nutrient and oxygen delivery to the tissues. To keep up with the demands of pregnancy, it is well known the maternal system increases blood volume to support the maternal tissues, (i.e., gastrointestinal tract, kidneys, skeletal muscle, etc.) as well as the growing reproductive tissues (i.e., uterus, mammary glands, etc.), which includes the conceptus. Therefore, adequate blood volume expansion during pregnancy is essential for fetal survivability (Thornburg et al., 2000).

The majority of research in blood volume expansion during pregnancy has focused on mid- to late pregnancy. In women, blood volume expansion during pregnancy is moderate during the first trimester, increases rapidly during the second, with only a slight increase in the third trimester (Pritchard, 1965). In cattle, plasma volume was increased throughout the pregnancy (Reynolds, 1953). However, these measurements were taken after the first trimester of pregnancy (Reynolds, 1953). In sheep, blood volume expansion during pregnancy has been debatable, with reports of blood volume expansion during pregnancy (Caton et al., 1975; Daniel et al., 1989), and small to no differences between pregnant and non-pregnant ewes (Metcalfe and Parer, 1966; Rumball et al., 2008). A drop in blood volume in day 20 and 40 of pregnancy was observed in swine (Anderson et al., 1970). However, blood volume in swine starts to increase at day 60 and throughout the rest of the gestation period (Anderson et al., 1970). Although, it was suggested that was due to the close confinement of the pigs in that study (Anderson et al., 1970).

The endocrine control theory of blood volume expansion suggests fetal influence on blood volume during pregnancy (Longo, 1983). Estradiol-17β is thought to increase maternal blood volume under the influence of dehydroepiandrosterone (DHEA) production, a hormone released from fetal adrenal glands (Longo, 1983). Estrogen, specifically E2, is formed in the placenta through the aromatization of DHEA and DHEA sulfate (Parker, 1999). The earliest DHEA can be detected is at 10 weeks of gestation in women (Parker, 1999). The plasma concentration of DHEA increases progressively through the second and third trimester of pregnancy and, along with it, estrogen increases (Parker, 1999).

In cattle, a recent study reported E2 plasma levels were positively correlated to estrous activity and estrous length (Lyimo et al., 2000). When measuring E2 plasma concentration 12 h before estrus and for 8 days afterwards, E2 plasma concentration was greater in fertile inseminations when compared to infertile inseminations (Erb et al., 1976). Based on this finding along with the effect of E2 increasing plasma volume during pregnancy, we suggest that the increase in E2 during estrus will increase blood volume and this eventually will increase the chances of the embryo to survive in the early stages of pregnancy.

Methods for measuring blood volume

Measuring blood volume has been a difficult task due to technical difficulties in obtaining an accurate measurement in addition to interpreting the results even if the measurements were accurate (Feldschuh and Enson, 1977). Historically, blood volume was measured through water dilution method or a bleed-out method (Feldschuh and Enson, 1977). However, these methods were very invasive and lethal. The criteria for an ideal blood volume tracer is to be 1) non-invasive; 2) does not change its chemical composition within the vascular space; 3) mixes completely within the vascular space; and 4) the concentration of the tracer can

be measured precisely (Feldschuh and Enson, 1977). Dye-dilution techniques can be used to measure blood volume less invasively (Feldschuh and Enson, 1977). Dyes such as brilliant vital red and Evans blue have been used successfully. However, it should be noted that when dyes are injected, a portion of the dye can be absorbed by the liver reticuloendothelial cells, which are a group of cells that sequester inert particles and vital dyes. This sequestration can lead to errors in blood volume measurements (Feldschuh and Enson, 1977). Radioactive isotopes can provide a more precise measurement (Feldschuh and Enson, 1977); however, this is not ideal for safety reasons. Unlike dyes, albumin within the circulation is not treated as a foreign body, thus albumin was also used to measure blood volume, specifically the plasma compartment (Feldschuh and Enson, 1977). However, albumin can transudate out of the circulation leading to error in the calculation (Feldschuh and Enson, 1977).

For the previous reasons, the determination of the mean body hematocrit seems like the best way to measure blood volume. Such a measurement is less invasive and does not require the introduction of any foreign substance to the blood. Hematocrit is a necessary parameter in measuring red cell volume (Gómez Perales, 2015). Hematocrit is defined as the proportion of the blood volume that consists of red blood cells (Gómez Perales, 2015). Despite the advantage of using hematocrit as an indicator for blood volume, hematocrit measurements can lead to errors when interpreted (Gómez Perales, 2015). For example, different body posture, upright vs supine, and capillary type, artery or vein vs narrow capillary, can lead to different measurement (Gómez Perales, 2015). However, this may not be the case in our study since all sample were taken in the same position and via the same capillaries.

Statement of the problem

Prenatal loss is a challenge that many livestock producers face and can result in a substantial economic loss. The loss can occur any time during the gestation period. However, most of the losses occur early after insemination making it difficult to detect. Pregnancy diagnosis techniques either direct, i.e., transrectal palpation and transrectal ultrasonography, or indirect, i.e., detection of progesterone or pregnancy associated factors, were developed to identify pregnant and non-pregnant animal to diminish the economic loss of the pregnancy loss. However, none of the available pregnancy detection methods fits the ideal pregnancy test criteria and none provide a solution for the problem of embryonic loss.

Blood volume increase is important to support the growing fetus with nutrients and oxygen. The increase in blood volume in mid- and late gestation is known in some mammalian species. However, information about blood volume at very early stages of pregnancy is lacking. An important theory about blood volume increase during pregnancy provides evidence that E2 production is a driver for this phenomenon. It is hypothesized that the rise of maternal E2 during estrus would increase plasma volume expansion that occurs during estrus.

The objectives of this thesis are to determine the relationship between blood volume during estrus and subsequent pregnancy rates in dairy cattle. We hypothesized that elevated E2 levels around the time of estrus will lead to an increase in blood volume, which will support the embryo increasing survival at early stages of gestation. We also, studied the effect of E2 on uterine cell proliferation in ovariectomized sheep with 24 h of E2 exposure in a model where plasma volume was measured.

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CHAPTER 2. THE EFFECT OF MATERNAL HEMATOCRIT ON FERTILITY AND EARLY EMBRYONIC LOSS IN DAIRY COWS

Abstract

Reproductive performance is one of the most important aspect of successful dairy farms. The hypothesis for the decrease in fertility in the last 40 years is because of increased milk production by dairy cows. Plasma volume increase during mid- and late gestation is important to support the fetus and sustain pregnancy. One factor to increase plasma volume during mid to late pregnancy is estradiol-17β (E2). Levels of E2 increase at estrus in all mammals. Currently, there are no available methods that allow us to predict the outcome of insemination. We hypothesized greater plasma volume levels at breeding will be related to greater chances of pregnancy success. The objective of the current experiment was to use hematocrit as a marker for plasma volume during estrus and early pregnancy in dairy cows, to determine if levels of hematocrit could predict pregnancy outcomes in dairy cows. Experiment 1 was conducted at the NDSU Dairy Research and Teaching Facility, while Experiment 2 was conducted with producers from 4 North Dakota dairies and 1 Wisconsin dairy. In Experiment 1, hematocrit was determined on day 0 (day of insemination), 5, 10, 12, 14, 15, 18, 20, 22, 24, 26, 28, 30, 60, 120, and 150 after estrus. In Experiment 2, hematocrit was only determined on the day of breeding (day 0). Animals were grouped into 4 pregnancy loss groups: Open: females that returned to estrus within 23 days after artificial insemination; Loss Group 1: females that returned to estrus between 24 and 30 days; Loss Group 2: females that were determined to be non-pregnant via ultrasonography or rectal palpation by 60 days; or Pregnant: females that were pregnant and remained pregnant through 150 days or beyond. In Experiment 1, hematocrit on days 0, 5, and 10 were decreased in cows from Loss Group 1 compared to Loss Groups 2, 3, and 4, which did not differ. When looking at

just the day of insemination, there was a tendency (P = 0.08) for cows that returned to estrus to have a greater hematocrit than cows that had achieved pregnancy, albeit some loss occurred in some cows. Using this information, for Experiment 2, only hematocrit on day of artificial insemination was evaluated. The Wisconsin farm did not determine the number of cows in the open group. The differential hematocrit levels at breeding were not observed across the Loss Groups as was observed in Experiment 1. Moreover, when data sets were combined, while there was a significant difference (P < 0.01), the differences were driven by farm, and not across Loss Groups. When hematocrit level was examined by pregnancy achievement, there was only one dairy that provided results similar to Experiment 1. When combined, there was no difference in hematocrit in cows that achieved pregnancy (i.e., Loss Groups 1, 2, and pregnant) compared to those that did not become pregnant (Open Group). When the dairy was analyzed by those that remained pregnant (i.e., Pregnant Group) compared to those that did not remain pregnant (i.e., Loss Groups 1, 2, and Open Group), there was no difference in hematocrit values (P ≥ 0.18). While physiological data still supports that plasma volume increase should influence pregnancy status, the use of hematocrit as a marker to determine plasma volume expansion at estrus, and its ability to predict pregnancy outcomes is not consistent in the dairy cow. Our studies do not support our hypothesis that we can predict this status with the use of hematocrit as a consistent marker for pregnancy success.

Key words: hematocrit, conception, dairy.

Introduction

In the 1980s fertilization rates in Holstein-Friesian dairy cows ranged from 90 to100%; however, because of many factors such as heat stress, reduced oocyte or sperm quality, this percentage dropped to 83% I the late 80s (Sreenan and Diskin, 1986; Sartori et al., 2009).

Despite this high fertilization rate, Holstein pregnancy rates are only 39% on average since the 80s (Badinga et al., 1985; Diskin and Morris, 2008). This variation between fertilization rate and conception rate is mainly due to the embryonic death. Pregnancy loss can cause serious economic loss ranging from $46 to $300 per cow per lactation (Fricke et al., 2016).

In order to diminish this effect and avoid extended calving interval, early pregnancy detection is very important to identify non-pregnant cows and rebreed them as soon as possible. According to Fricke and coworkers (2016), the ideal pregnancy test for dairy cows should fulfill these criteria: 1) high sensitivity (i.e., correctly identify pregnant animals); 2) high specificity (i.e., correctly identify nonpregnant animals); 3) inexpensive to conduct; 4) simple to conduct under farm conditions; 5) ability to determine pregnancy status at the time the test is performed; and 6) the ability to determine the pregnancy status with the least physical interaction. Most pregnancy detection methods that have been implemented in modern dairy can accurately determine the state of pregnancy as early as 30 days after insemination (Fricke et al., 2016). However, none of these methods can predict early embryonic loss.

Estradiol-17β (E2) plasma concentrations around estrus have been related to fertility in cows. When comparing estrogen levels in fertile to infertile cows, plasma E2 levels were greater in fertile cows particularly 12 h before estrous and for 8 days afterwards (Erb et al., 1976). Additionally, E2 may increase blood volume during mid pregnancy (Longo, 1983). Blood volume increase during mid and late gestation is important to support the fetus with nutrient and oxygen. A short duration of E2 (i.e., 24 hours) tended to increase plasma volume in ovariectomized ewes (Vasquez-Hidalgo et al., 2019).

Additionally, plasma volume levels were greater in ewes carrying twins comparing to ewes carrying singletons and we were able to detect the differences as early as 20 days after

insemination (Vasquez and Vonnahme, unpublished). Furthermore, when compared plasma volume in pregnant and non-bred ewes on day 10 post estrus, there was a increase in pregnant animals compared to non-pregnant animals (Chapter 3).

Ideally, E2 levels can be an indicator for fertility. However, the increased of blood flow to the liver in dairy cattle causes increases in the metabolic clearance rate of the steroidal hormones including E2 and progesterone (Sangsritavong et al., 2002), making it an unreliable marker for fertility.

These data led to our hypothesis that E2 during estrus would increase plasma volume, thus decreasing hematocrit; therefore, hematocrit could be used as a marker for pregnancy success. The objective was to determine if hematocrit could be a reliable marker for fertility in dairy cattle.

Materials and methods

The animal portion of this experiment was approved by the Institutional Animal Care and Use Committee at North Dakota State University (A15081).

Experiment 1

This experiment was conducted at the NDSU Dairy Research and Teaching facility. Initially, 74 Holstein heifers and cows were bled on the day of breeding (either due to standing estrus or by synchronization; day 0), and days 5, 10, 12, 14, 15, 18, 20, 22, 24, 26, 28, 30, 60, 120, and 150 after estrus. Blood samples were collected from the coccygeal vein with a 20 gauge, 2.5 cm needle into vacuum tubes containing ETDA (Becton Dickinson, Franklin Lakes, NJ). Blood samples were immediately inverted and placed on ice until centrifugation via a micro-capillary centrifuge for two minutes (International Equipment Company, Nashville, TN). Microhematocrit capillary tubes (Globe Scientific Inc., Dayton, NJ) were used to assess the

hematocrit within each blood sample by measuring the packed red cell length and total sample length with a digital caliper. To calculate percentage hematocrit, the packed red cell length was divided by the total sample and multiplied by 100.

To assign to a pregnancy loss group, all females that returned to estrus within 23 days after artificial insemination were placed into loss group 1 (n = 9), females that returned to estrus between 24 and 30 days were placed into loss group 2 (n = 8), females that were determined to be non-pregnant via ultrasonography or rectal palpation by 60 days were placed into loss group 3 (n = 29) and females that were pregnant and remained pregnant through 150 days were placed into loss group 4 (n = 28).

Experiment 2

Due to the information that was obtained from Experiment 1, dairies from North Dakota and Wisconsin were enrolled in the study and only blood samples were obtained on the day of breeding (Table 3). Blood was processed similarly to Experiment 1. At all locations, cows were housed in free-stall barns during their lactating period and were fed a total mixed ration supplemented with concentrate, based on their milking stage. Cows have had free access to water.

Table 3. Dairies from ND (A, B, C, and D) and WI (E) that were used to determine how hematocrit at estrus predicts pregnancy outcomes.

Dairy	A	B	C	D	E
n	33	208	96	84	103
Bred by:	1 natural heat; 32 synchronization	35 natural heat; 173 synchronization	6 natural heat; 90 synchronization	35 natural heat; 49 synchronization	Synchronization
Pregnancy diagnosis by:	Blood (35d) Rectal (65)	Blood (35d) Rectal (65)	Ultrasound (35d) Rectal (65)	Rectal (35d) Rectal(65)	Ultrasound (35d) Rectal (65)
Loss groups	Open, 1, 2, Preg	Open, 1, 2, Preg	Open, 2, 3, Preg	Open, 1, Preg	1, 2, Preg

Pregnancy results were obtained from each dairy. Cows were divided into 2 groups that represent their pregnancy status. Open Group; cows that were diagnosed non-pregnant by returning to estrus within 17 to 23 days after artificial insemination, or by using different methods of pregnancy diagnosis such as rectal palpation (Dairy D), ultrasound (Dairy C) or blood test (Dairies A and B). Pregnant Group; cows that were diagnosed pregnant and carried their pregnancy to full term.

Statistical analysis

In experiment 1, data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc, Cary, NC). The model included the fixed effect of loss group and random effect of cow. Day was a repeated measure. When the overall determination of hematocrit levels at breeding within loss group was performed, MIXED procedures of SAS were used, and only day 0 hematocrit values were assessed. In experiment 2, data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc, Cary, NC, USA). The model included the fixed effect of treatment and the random effect of the cow. The month of collection and method of heat detection were the dependent variables. Means were separated using the LSMEANS procedures. Significant was declared at $P \leq 0.05$, and tendencies were declared from $0.05 < P \leq 0.10$.

Results and discussion

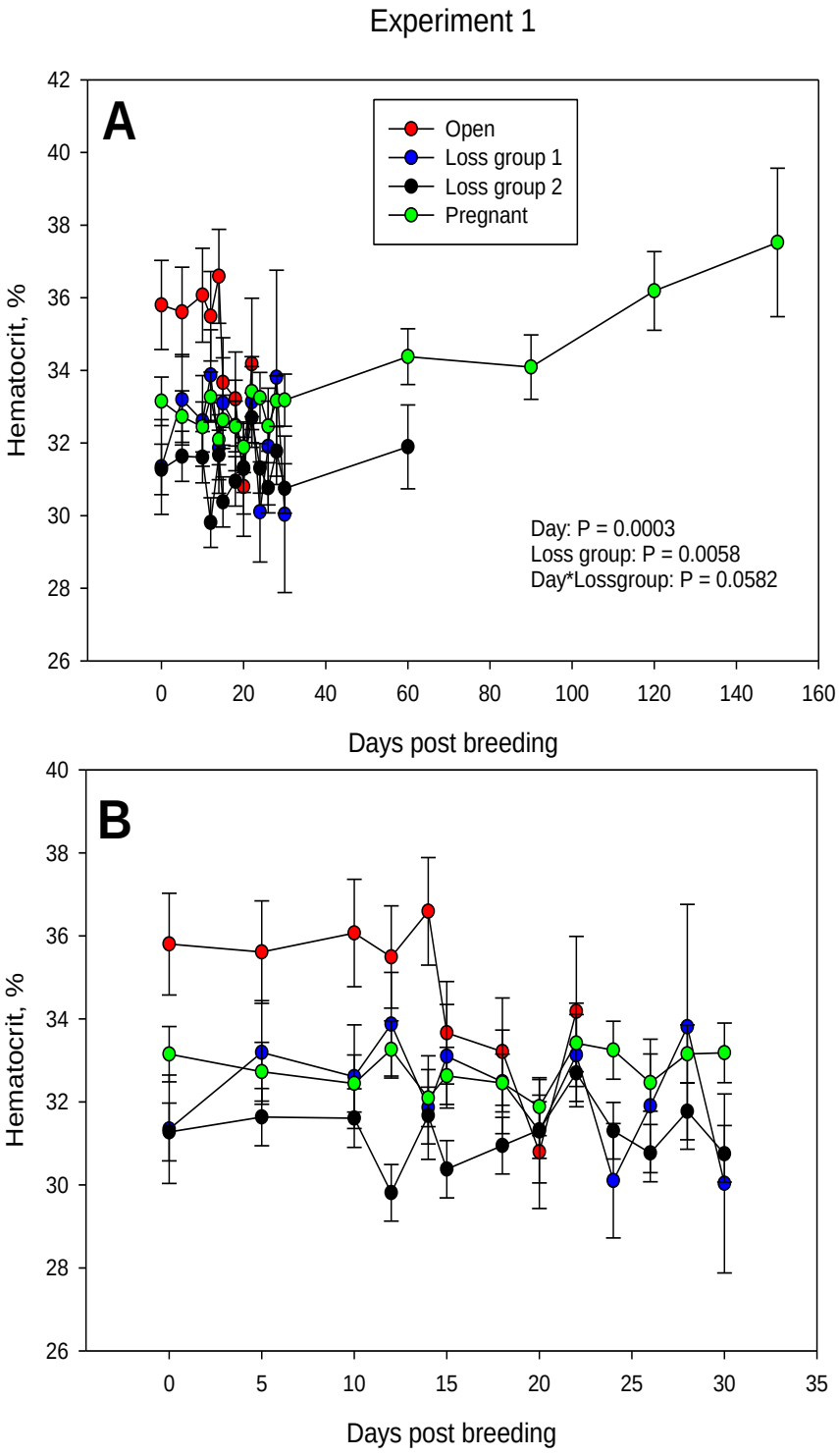

Figure 2. Hematocrit changes within loss group from day 0 to 150 after breeding.

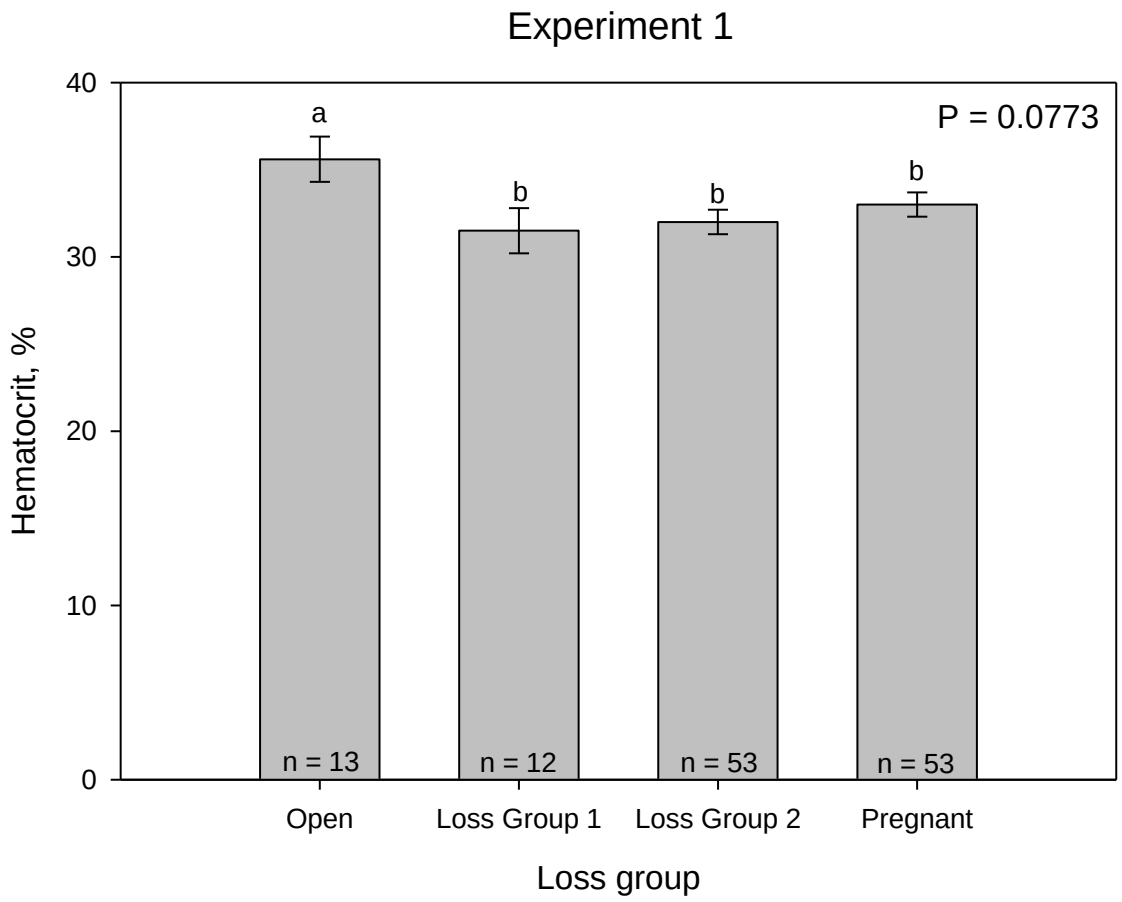

Figure 3. Hematocrit from females on the day of breeding from Experiment 1.

In Experiment 1, we noted that hematocrit differences between loss groups could be noted as early as day 0 and day 5. Thereafter, there was no effect of loss group on hematocrit, except for when comparing 3 vs 4 on day 60. When we further evaluated the impacts of hematocrit collected on day 0, we determined that hematocrit collected on the day of breeding tended to predict future success in pregnancy outcomes. In figure 2, we see that cows that returned to estrus within 23 days had increased hematocrit compared to those that were considered pregnant. However, there was no distinction between those cows that were pregnant but lost their calves at some point along gestation, compared to cows that remained pregnant past 150 days. Based on the information from Experiment 1, blood samples collected at the time of insemination in Experiment 2. Data from Experiment 1 supported our hypothesis, but the number

of experimental units was low. In order to confirm out findings, additional dairies were investigated.

Table 4. Experiment 2. Hematocrit analyzed within a dairy based on insemination outcome.

Farm	Open group	Loss group 1	Loss group 2	Preg group	P value
A	30.92 ± 0.57^{a}	28.56 ± 0.44^{b}	34.37 ± 1.71^{c}	29.98 ± 0.61^{a}	0.002
B	30.35 ± 0.37^{x}	30.30 ± 0.34^{x}	34.31 ± 1.79^{y}	30.74 ± 0.35^{x}	0.13
C	30.89 ± 0.80	31.95 ± 0.75	30.22 ± 2.22	31.02 ± 0.62	0.71
D	32.44 ± 0.56	NA	32.24 ± 0.63	31.51 ± 0.48	0.41
E	NA	31.09 ± 0.46	33.47 ± 1.58	32.20 ± 0.57	0.16

[ab]LSMeans ± SEM within a row with different superscripts differ, $P \leq 0.05$
[xy]LSMeans ± SEM within a row with different superscripts differ; unprotected F test; $P \leq 0.05$
NA: Not applicable, information was unknown.
loss group 1: females that returned to estrus within 23 days, loss group 2: females that returned to estrus within 23-30 days, loss group 3: females that were determined to be non-pregnant by d 60, loss group 4: females that were pregnant and remained pregnant through 150 days

The data across all ND farms were combined and statistical analyses were performed. The dairy in WI was excluded as it did not contain Group 1 information (this was the only Loss Group that was different in Experiment 1). Because there was a difference in the determination of pregnancy to determine loss groups, data were grouped into the following: Group 1 was still considered animals that returned to estrus in 23 days; Groups 2 and 3 were merged into Loss Group 1-2 indicating cows lost their pregnancy between days 30 and 60; and Group 4 remained those cows that were determined to be pregnant past day 60. Summary statistics are shown in Table 5.

Table 5. The number of cows from ND dairies utilized in the merged data set.

Farm	Open	Loss Group 1-2	Pregnant
A	9	16	8
B	56	74	63
C	23	29	38
D	26	20	37

Table 6. Experiment 2. Data analyzed across all ND dairies.

Farm	Open	Loss group 1-2	Pregnant
A	30.92 ± 1.02^{ab}	28.92 ± 0.77^{a}	29.98 ± 1.08^{a}
B	30.50 ± 0.41^{a}	30.36 ± 0.36^{b}	30.73 ± 0.39^{a}
C	30.89 ± 0.64^{a}	31.77 ± 0.57^{c}	31.02 ± 0.50^{a}
D	32.44 ± 0.60^{b}	32.24 ± 0.69^{c}	31.62 ± 0.50^{a}

There are no differences within a farm. ^{abc}LSmeans ± SEM within a Loss Group with different superscripts differ, $P \leq 0.05$.
Hematocrit LSmeans ± SEM from each loss group nested within a dairy; $P = 0.0089$.

Table 7. Females that achieved pregnancy compared to those that returned to estrus (i.e. Achieved pregnancy = Loss groups 2, 3 and 4 vs Did not achieve = Loss group 1).

Farm	Achieved pregnancy	Did not achieve pregnancy	P value
All dairies	31.05 ± 0.30	31.07 ± 0.16	0.95
A	**30.92 ± 0.66**	**29.27 ± 0.41**	**0.04**
B	30.34 ± 0.37	30.57 ± 0.26	0.58
C	30.89 ± 0.80	31.35 ± 0.47	0.62
D	32.44 ± 0.56	31.77 ± 0.38	0.33
E	NA	NA	NA

NA: W dairy did not record data to categorize Loss Group 1.

Our hypothesis was not supported by the data in Experiment 2. With the exception of one dairy, Dairy A, hematocrit levels did not change between cows that were found pregnant and cows that failed to achieve pregnancy. Additionally, amongst all dairies, Dairy A had only 33 animals involved, which did not give enough power to support the results.

If blood volume is changed, the reason for not detecting difference in blood volume may be because it is too early to detect changes in plasma volume. The increase in blood volume in pregnant women was not detected until the sixth week of pregnancy (Bernstein et al., 2001). Another reason for not identifying difference in plasma volume is hematocrit maybe too crude of a measure for detecting plasma volume. A previous study in humans used different and more accurate techniques to detect changes in plasma volume by measuring creatinine clearance rate, volume-dependent hormones, hemodilution-related variables and ultrasonic vascular dimension (Duvekot et al., 1995). The difference between our findings in cattle vs sheep (citation) may be attributed to the increase of the liver blood flow in dairy cattle that causes increases in the metabolic clearance rate of the steroidal hormones including E2 and progesterone (Sangsritavong et al., 2002), diminishing the effect of E2 on plasma volume.

Additionally, hematocrit in cattle can change due to other factors. Hematocrit levels were lower in older compared to younger Guernsey cows (Lane and Campbell, 1969). We did not record parity in the current studies. Also, beef cattle that suffer from heat stress had lower hematocrit levels than cattle that were housed in a control environment (Biggers et al., 1987). However, bodyweight and milk production levels did not affect hematocrit levels (Lane and Campbell, 1969).

In conclusion, packed cell volume did not differ between outcome groups and were not able to predict early embryonic losses. This might be due to one of two reasons; 1) changes in plasma volume is cattle is too early to detect at the day of insemination, 2) hematocrit levels may not be the best indicator of changes in plasma volume. Incorporating more accurate method to detect changes in plasma volume maybe more beneficial. Also, tracking the changes in plasma volume over the course of the couple of weeks following insemination can help us understand

more about the mechanism of the increase of blood volume and whether it is related to early embryonic loss or not.

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CHAPTER 3. HEMATOCRIT AT ESTRUS AND ITS PREDICTION ON FERTILITY AND FECUNDITY IN EWES

Abstract

Early pregnancy is critical due to the major developmental changes that occur such as embryonic organogenesis and the formation of the placenta. Overall, most of the embryonic losses in ewes occur in the first 18 days after fertilization. We hypothesized that E2 from ovulatory follicles would increase blood volume at estrus. Whiteface ewes (n = 124) were placed with a ram wearing a marking harness to determine breeding. A blood sample from the jugular vein was collected at the day of breeding and hematocrit levels were recorded. Hematocrit levels in the ewes that failed to conceive were lower (P = 0.02) than the ewes that had singletons (33.8 vs 35.7 ± 0.4%). Hematocrit levels proceeding estrus in ewes carrying twins were not different than ewes that failed to conceive or the ewes carrying twins. The data in this study did not support our hypothesis and suggest that ewes that failed to conceive had greater plasma volume near estrus compared to non-pregnant status.

Key words: blood volume, conception, ewe

Introduction

Early embryonic death is a common problem in all mammalian species. Factors leading to embryonic loss can be genetic, maternal, or environmental (Hanly, 1961; Wilmut et al., 1986). In ewes, embryonic loss is estimated at 28.6% (Hulet et al., 1956) with most embryonic loss occurring in the first 18 days after fertilization (20.4%) (Hulet et al., 1956). This was confirmed by Dixon et al. (2007) where embryonic loss was reported to be 28% before d 25 of pregnancy.

Follicular and circulating E2 concentrations have been linked to fertility in cows. Oocytes from ovulatory follicles with greater concentrations of E2 were more likely to develop to the

blastocyst stage after in vitro maturation and fertilization were performed (Mermillod et al., 1999). Increased preovulatory circulating serum concentrations of E2 resulted in increased pregnancy rates (Perry et al., 2005, 2007; Lopes et al., 2007; Brides et al., 2010). It may be unlikely that the increased E2 concentrations impact the oocyte directly as the addition of E2 to IVM media is either detrimental or has no effect on the early embryo (Beker-van Woudenberg et al., 2004, 2006). It was hypothesized that preovulatory concentrations of E2 were having a greater impact on pregnancy success due to gamete transport, regulation of the uterine environment, or both (Geary et al., 2013).

In sheep, eliminating the preovulatory E2 surge resulted in decreased uterine weight, uterine protein synthesis, and embryo survival following embryo transfer (Perry et al., 2009). During later gestation, increased E2 concentrations(d 45 and d 65 of pregnancy) was thought to be responsible for lower embryonic loss from d 45 or 85 to parturition (Dixon et al., 2007).

Our laboratory has recently demonstrated that an acute exposure (i.e. 24 h) to E2 tended to increase plasma volume in ovariectomized ewes (Vasquez-Hidalgo et al., 2019), indicating that increased follicular E2 concentrations may enhance vascular volume and potentially blood flow to the reproductive tissues. When plasma volume increases, hematocrit levels decrease. Our laboratory has also reported that hematocrit levels were decreased as early as day 20 after conception in ewes carrying twins compared to ewes carrying singletons (Vasquez-Hidalgo et al., unpublished). Moreover, mid-luteal phase (day 10 after estrus) ewes that were not bred had increased hematocrit concentrations compared to ewes that were pregnant on day 20 (Vasquez and Vonnahme, unpublished). It is currently unknown if hematocrit concentrations at estrus are predictive of reproductive success in ewes.

The objective of the current study was to determine if hematocrit around the time of estrus was predictive of fertility and fecundity in sheep. We hypothesized that ewes achieving a successful pregnancy would have decreased hematocrit at estrus compared to ewes that returned to estrus or were diagnosed as non-pregnant by ultrasound on day 35 after estrus. Moreover, we hypothesized that ewes that would carry twins would have decreased hematocrit compared to ewes that would carry singletons.

Material and methods

Animals

This project was approved by North Dakota State University (NDSU) Animal Care and Use Committee (#A18009). Nulliparous whiteface ewes (n = 89) were housed at the Animal Nutrition and Physiology Center at NDSU. All ewes met their nutritional requirements as described in the NRC (2007). The ewes were housed with intact rams (n = 3) that were equipped with a marking harnesses with similar colored crayons and breeding marks were recorded once daily. The crayon on the ram harness was checked and changed as needed. After 17 days, the color of the crayon was changed in order to determine if a ewe returned to estrus. When ewes had a fresh mark on their back, a 10 mL jugular blood sample was collected via vacuum tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ) to determine hematocrit. After collection, hematocrit was determined using microhematocrit capillary tubes (Unico micro-hematocrite centrifuge, Dayton, NJ) and centrifuged for two minutes. Length of red blood cells and total sample volume were measured with digital calipers. The length of RBCs was divided by the total sample and multiplied by 100 to obtain hematocrit percentage. It was recorded if a ewe that had been previously marked returned to estrus (i.e., presence of a new mark from the rams). All ewes that did not return to estrus were restrained to determine pregnancy status and

fetal enumeration via ultrasonography (Aloka Prosound Alpha 6) 30 to 40 days after breeding marks were observed. Non-pregnant ewes were returned to the pen with the ram, and pregnant ewes were removed from the pen and housed separately.

Statistical analysis

Ewes were categorized into 2 categories: 1) pregnancy status (i.e. pregnant vs. not pregnant) and 2) fate after breeding (i.e. returned to estrus in 17-20 days; did not return to estrus and was non-pregnant at ultrasound; was pregnant at ultrasound). When characterizing pregnancy status, ewes were further categorized into those carrying singleton or twins. Data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc, Cary, NC, USA). Ewe was the experimental unit. The model included the fixed effect of pregnancy status, as well as fate after breeding and the random effect of the ewe. Variable of interest was hematocrit at estrus. One hematocrit value was removed as it was an outlier and was 3 standard deviations from the mean. Means were separated using the LSMEANS procedures. Significance was declared at $P \leq 0.05$, and tendencies were declared from $0.05 < P \leq 0.10$.

Results and discussion

Table 8. The difference between hematocrit levels in non-pregnant ewes vs ewes carrying singleton and twins.

	Pregnancy Status		
	Not pregnant (n = 62)	Singleton (n = 47)	Twin (n = 7)
Hematocrit, %	33.79 ± 0.37^{a}	35.46 ± 0.43^{b}	33.54 ± 1.11^{ab}
Range	26.21 – 39.69	27.57 – 43.59	29.95 – 47.93

Overall P value = 0.01; ^{ab}LSMeans ± SEM differ; P = 0.01

Ewes that were diagnosed as non-pregnant at the time of ultrasonography had a decreased (P = 0.01) hematocrit compared to ewes carrying singletons, with ewes carrying twins being intermediate (Table 8). Interestingly, hematocrit just proceeding estrus in ewes that carried twins

did not reach significance ($P < 0.05$) compared to ewes carrying singletons ($P = 0.11$). If the non-pregnant ewes were removed from the data set to compare hematocrit levels in just the pregnant ewes, the P value decreases to $P = 0.09$. This is similar to the data from Vasquez et al., (2019) where on day 20 of gestation, and when calculated as area under the curve, ewes carrying twins had decreased hematocrit compared to ewes carrying singletons. The data in the current study did not reach significance, which may be due to the low number of ewes carrying twins in the study. The data do not support our hypothesis that hematocrit would be reduced at estrus in sheep that become pregnant compared to those that lose their pregnancy (or do not conceive) by the time of pregnancy diagnosis (Table 9).

Table 9. The difference between hematocrit levels in non-pregnant ewes vs pregnant ewes.

	Pregnancy status		
	Non-pregnant (n = 62)	Pregnant (n = 54)	P -value
Hematocrit, %	33.79 ± 0.38	35.21 ± 0.40	0.01

There were 14 ewes who had hematocrit recorded at least 2 times where they had a time where they returned to estrus within a 17 to 20 day time frame, and then was diagnosed as pregnant. Of those 14 ewes, only 3 demonstrated a decrease in hematocrit, while 11 exhibited an increase in hematocrit (Figure 3.1). This information supports the data in Table 3.2 demonstrating that in the ewe, increased hematocrit just proceeding estrus may be a biomarker for fertility.

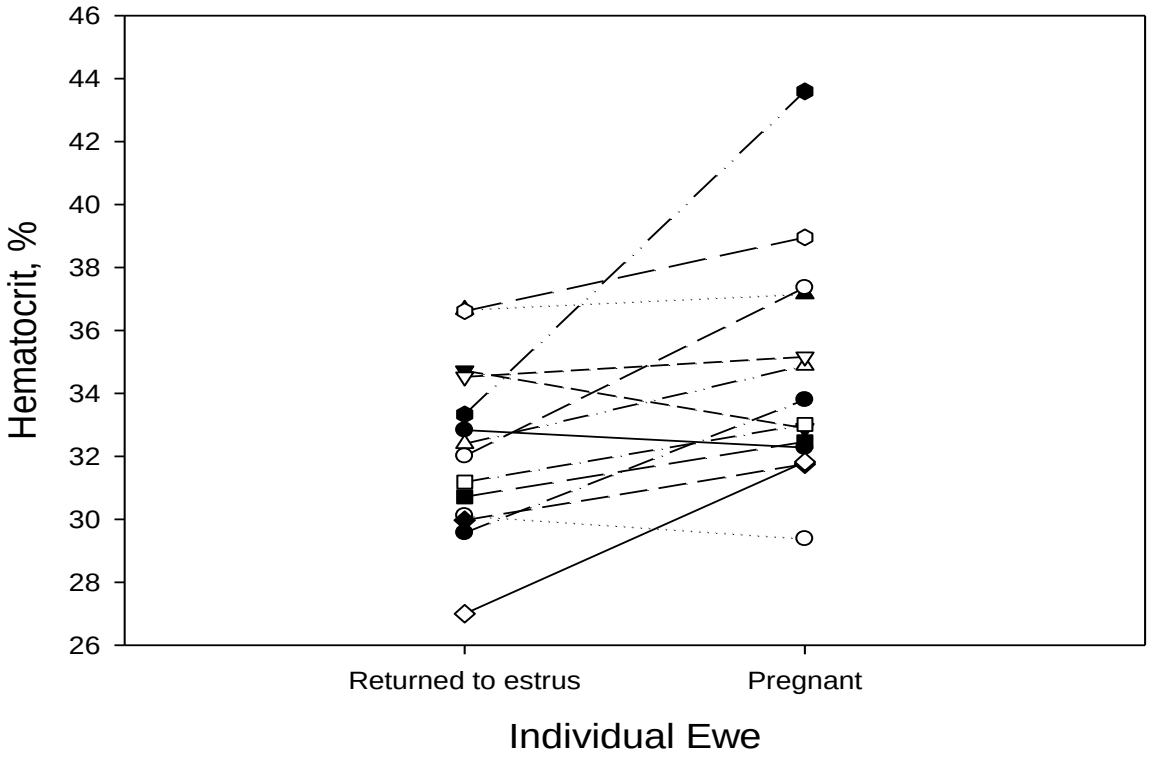

Figure 4. Hematocrit of pregnant ewes and ewes that returned to estrus.

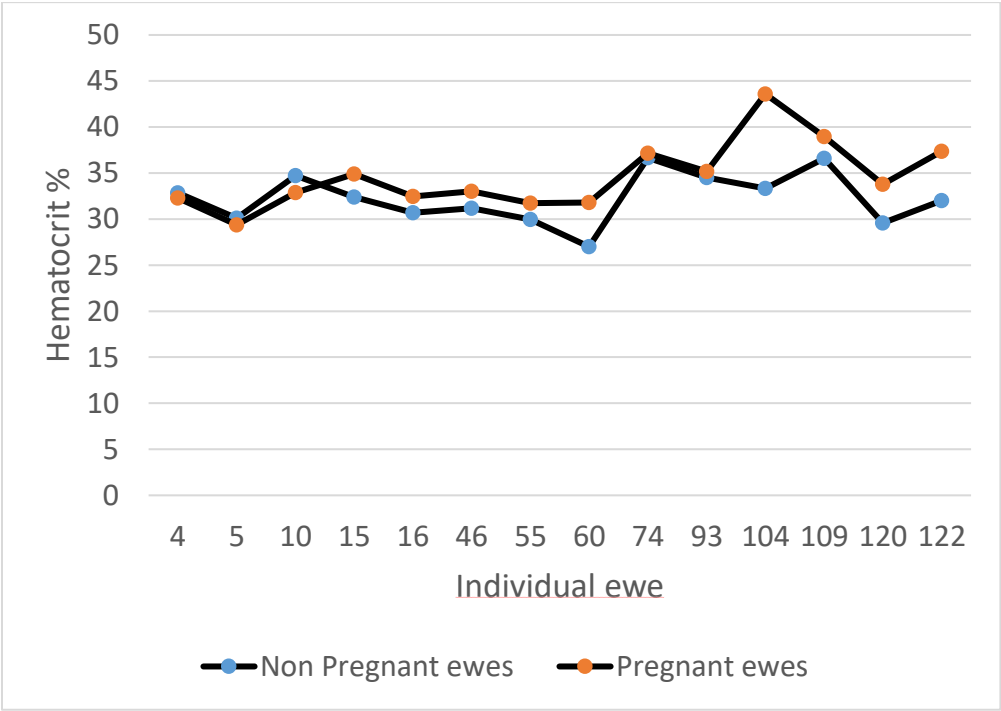

Figure 5. Hematocrit % difference between pregnant and non-pregnant ewes.

We did not determine circulating E2 concentrations in the current study, however, it is assumed that E2 concentrations would not be at peak concentrations as blood samples were taken after estrus as marked by the rams. Since ewes were checked once per day for markings, the ewes could have up to a 24 hour range in their time since bred. In spite of this, it is interesting that hematocrit is opposite of what was hypothesized. We have previously reported that plasma volume increased after 24 hr of pharmacological doses of E2 (Vasquez et al., 2019). This is supported by a study where infusion of E2 in ovariectomized ewes led to 27% increase in whole blood volume (Ueda et al., 1986). The increase in the blood volume was attributed to the increase in the plasma volume by 27% (Ueda et al., 1986).

One study found a tendency for women who did not conceive in this study to have greater estimates of plasma volume over the course of menstrual cycle (Bernstein et al., 2001). Although, this study did not have sufficient power to support their result due to the small number of the female participants (Bernstein et al., 2001).

The increase of E2 from proestrus to estrus enhances angiogenesis (Johnson et al., 1996). Angiogenesis takes place in the uterine endometrium during the transition from diestrus to estrus to prepare the uterus for the embryonic implantation (Yasuda et al., 1998). Estrogen is thought to enhance the angiogenesis process through upregulating the erythropoietin production, which is a key factor for regulating erythropoiesis (Yasuda et al., 1998). This can provide an explanation for our finding in this experiment. The increase in the hematocrit at the time of estrus maybe temporary and necessary to the erythropoiesis process. An increase in the plasma volume can start in later stages of pregnancy.

Although data obtained from this study did not support our hypothesis, the fact that there was a difference in hematocrit levels between ewes that successfully maintained their pregnancy

and the ewes that failed to maintain pregnancy may lead to new applications to predict fertility in ewes. The possible 24 hours' time difference between when the breeding mark took place and when the blood sample was taken could have an impact on the hematocrit levels. Further investigation to build up a timeline for the changes in plasma volume and ovarian hormones throughout the various stages of pregnancy might give us a better understanding for the changes that happen in the mother and how it would affect the fetus.

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CHAPTER 4. THE EFFECT OF IN VIVO ESTROGEN-TREATMENT ON UTERINE CELL PROLIFERETION IN OVARIECTOMIZED ROMANOV EWES

Abstract

Embryonic death in sheep may occur in the pre-implantation period due to deficiencies in uterine function. Ovarian steroids play an important role in preparing the uterus for implantation by enhancing histotroph and altering endometrial cell size and proliferation. We hypothesized that in vivo estradiol-17β (E2) treatment of ovariectomized ewes will result in enhanced uterine cell proliferation. Therefore, our objective was to determine the effects of 24 h of E2 exposure on cell proliferation in uterine compartments. At least 30 d after ovariectomy, Romanov ewes (n = 15) received silastic implants containing 0 mg (controls; n = 7) or 100 mg of E2 (n = 8) at the axillary region. At tissue collection 24 h later, uterine cross-sections were fixed in formalin followed by immunohistochemical localization of Ki67 (a marker of proliferating cells). Tissue sections were then counterstained with fast red to visualize cell nuclei. Images of luminal epithelium and endometrial stroma (5 areas each/tissue section) were analyzed to determine the labeling index (LI) (proportion of proliferating cells, based on Ki67 protein detection). Images of endometrial glands and myometrium were not analyzed because labeling index was very low. Ki67 was detected in cell nuclei in all uterine compartments including luminal epithelium, endometrial stroma and glands, and myometrium. Cell proliferation in the luminal epithelium was greater ($P = 0.02$) in E2-treated than control ewes (6.55 vs 1.2 ± 1.75%) and stromal cells tended to be greater ($P = 0.08$) in E2-treated than control ewes (0.59 vs 0.37 ± 0.06%). Thus, after 24 h of E2-treatment, cell proliferation in uterine luminal epithelium and stroma was enhanced compared to controls. These data indicate E2 control uterine cell proliferation in highly

fertile breeds and emphasize the importance of estrogens in regulation of uterine growth and function.

Key words: estradiol, proliferation, ewes.

Introduction

Ovarian steroids, estrogen and progesterone, play an important role in preparing the uterus for successful implantation and support embryonic growth and placentation (Johnson et al., 1997c; Koos, 2011). These steroids in various combinations and durations of exposure increase uterine cell proliferation, vascular growth, and microvascular permeability (Reynolds, 1949; Reynolds et al., 1998). The proliferative response of the uterine cells to estrogen may be due to the activation of protooncogenes which encode nuclear regulatory protein and the enhanced expression growth factors and their receptors in addition to cAMP- independent protein kinase (Murphy and Ghahary, 1990). Many studies were conducted in order to quantify the uterine cell proliferation that happen in response to E2 (Johnson et al., 1997c; Reynolds et al., 1998). These were performed by injecting the ewes with bromodeoxyuridine (BrdU) -a thymidine analogue- and observing the percentage of cells exhibiting nuclear incorporation of BrdU (Johnson et al., 1997c). Furthermore, Reynolds and coworkers were able to establish the time-course of uterine cell growth, cell proliferation, and microvascular developments throughout a 72 hour time frame after implanting E2(Reynolds et al., 1998). The rate of uterine proliferation was low through 4 h of exposure to E2 with slight increase at 8 h. However, after 24 h there was a moderate increase in proliferation in the glandular epithelium and a massive increase in the luminal epithelium and stroma was observed (Reynolds et al., 1998). The Romanov breed is well known for its prolificacy (Ben Said et al., 2007). The response of the multi-ovulating Romanov ewe to E2 is different than other single-ovulatory breeds. While

estrous behavior can be induced in Romanovs with smaller dose of E2, much greater levels of E2 are required to induce the luteinizing hormone surge comparing to single-ovulatory breeds (Ben Said et al., 2007). It is unknown if the Romanov uterus would respond similarly to other breeds of sheep when exposed to estrogen. We hypothesized that treating ovariectomized Romanov ewes with E2 will increase uterine cell proliferation.

Materials and methods

Animals

Animals care and use was approved by the Institutional Animal Care and Use Committee at North Dakota State University (#A17009). Multiparous Romanov ewes (n = 15) were housed individually to monitor their feed intake at the Animal Nutrition and Physiology Center at North Dakota State University. All ewes received 100% of their nutritional requirement as described in the NRC (2007). All the ewes were ovariectomized and were allowed a recovery period of at least thirty days. Thereafter, Romanov ewes (n = 15) received silastic implants containing 0 mg (controls; n = 7) or 100 mg of E2 (n = 8) at the axillary region as was described by O'Neil et al. (2009).

Sample collection

Twenty-four hours after the implant was placed, ewes were euthanized, the uterus was obtained, weighed, and uterine cross-sections were fixed in 10% neutral buffer formalin followed by immunohistochemical localization of Ki67 (a marker of proliferating cells; mouse monoclonal antibody from Vector Laboratories, Burlingame, CA). Tissue sections were then counterstained with fast red (Sigma, St. Louis, MO) to visualize cell nuclei. Images of luminal epithelium and endometrial stroma (5 areas each/tissue section) were analyzed using a software

program (Image Pro 9.2) to determine the labeling index (percentage of proliferating cells out of total cell number per selected area; Figure 4.1).

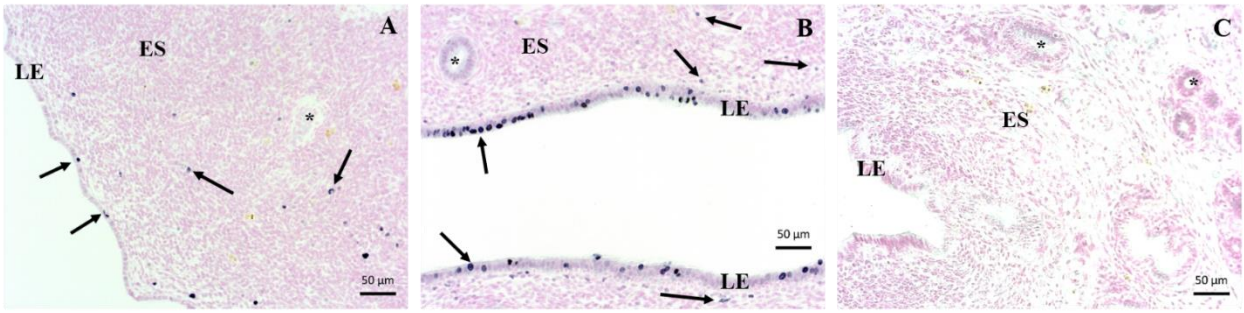

Figure 6. Representative images of Ki67 immunohistochemical staining (black color) in ovine uterus from non-treated control (A) and E2-treated (B) ewes. ES: endometrial stroma; LE: luminal epithelium; *: endometrial glands. Arrows points on proliferating cells (black) in LE and ES. Note a lack of staining in endometrial glands. (C) Control staining where primary antibody was omitted. Pink color indicates nuclei stained with fast red.

Statistical analysis

Data were analyzed using the GLM procedure of SAS (SAS Inst. Inc, Cary, NC, USA). Ewe was considered the experimental unit for all variables. The model included the fixed effect of treatment and the random effect of the ewe. Means were separated using the LSMEANS procedures. Significant was declared at $P \leq 0.05$, and tendencies were declared from $0.05 < P \leq 0.10$.

Results and discussion

Our data confirm the role of E2 in uterine cell proliferation, especially the proliferation on the luminal compartment of the uterine stroma similar to the mixed breeds that were used in the study that was conducted by Reynolds and coworkers (Reynolds et al., 1998).

Previous attempts to quantify cell proliferation in ewes using BrdU as proliferation marker (Johnson et al., 1997a; Reynolds et al., 1998). However, when comparing BrdU to Ki-67 within brain cells in Wistar rats, in a parallel study, the latter shows 50% great values (Kee et al., 2002). This was attributed to the ability of Ki-67 to be incorporated to into DNA during all the

phase of mitotic process, while BrdU can only be incorporated to DNA during the S- phase of mitosis (Kee et al., 2002).

Our data showed that uterine fresh weight and uterine weight/BW were greater ($P < 0.05$) in the ewes that received E2 treatment compared to the control group (Table 4.1). Similar results in different breeds of sheep were obtained from different studies (Johnson et al., 1997a; Reynolds et al., 1998). These results can be explained by the increase in the uterine blood flow in the animals that receives E2 (Magness et al., 1998; Magness et al., 2005)

Table 10. The difference between BW, uterine weight, uterine weight/BW in E2-treated ewes vs control ewes

Item	E2	CON	*P* – value
BW (kg)	46.83 ± 2.13	44.58 ± 2.64	0.50
Uterine weight (g)	27.98 ± 3.44	16.51 ± 2.38	0.02
Uterine weight/BW (g/kg)	0.59 ± 0.06	0.37 ±0.04	0.01

The proliferation marker Ki67 was detected in cell nuclei and in all uterine compartments including luminal epithelium, endometrial stroma and glands, and myometrium. A previous study was able to analyze the LI in myometrial cells (Johnson et al., 1997a). However, in our experiment, images of endometrial glands and myometrium were not analyzed because LI was very low. In the endometrium, percentage of cell proliferation in the luminal epithelium was greater ($P = 0.02$) in E2-treated than control ewes and stromal cells tended to be greater ($P = 0.07$) in E2-treated than control ewes (Table 4.2). Thus, after 24 h of E2-treatment, cell proliferation in uterine luminal epithelium and stroma was enhanced compared to controls (Table 4.2).

Table 11. Percentage of proliferating cells out of total cell number/selected area in luminal epithelium and endometrial stroma in E2-treated vs control ewes

	Treatment		
	E2	Control	*P* - value
Epithelium Labeling index (%)			
Luminal epithelium	6.55 ± 1.75	1.20 ± 0.64	0.02
Endometrial stroma	0.57 ± 0.22	0.09 ± 0.04	0.07

Labeling Index: proportion of proliferating cells, based on Ki67 protein detection

The stromal layer of the endometrium plays an important role in pregnancy success by hosting the blood supply necessary for implantation and growth of the embryo. The results of this study along with previous data (Johnson et al., 1997b; Reynolds et al., 1998) suggest that the luminal tissue compartment of the uterine stroma might consider the glandular compartment of the ovine endometrium.

In conclusion, E2 increased uterine cell proliferation and uterine weight in ovariectomized Romanovs as expected. However, further investigation is required to be able to compare the difference in E2 response between high fertile and less fertile breeds. In order to accomplish this, all breeds would receive the same E2 treatment in the same study and cell proliferation should be detected using the same proliferation marker.

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CHAPTER 5. CONCLUSION

Our hypothesis was that the increase in E2 around estrus will increase plasma volume and consequently improve pregnancy outcomes. However, our data from both the dairy cattle and sheep experiment did not support this hypothesis. In fact, hematocrit levels were greater in sheep that had successful pregnancies.

The literature and data from our lab (Vasquez-Hidalgo et al., 2019) support that E2 increases blood volume in later stages of pregnancy. However, this may not be the case at estrus. Other data showed that E2 increases erythropoiesis, the production of red blood cells, around estrus (Yasuda et al., 1998). Therefore, we suggest studying the effect of E2 on blood volume and blood components more closely throughout the different stages of pregnancy. Such data can be very helpful in understanding the relationship between E2 and blood volume and components.

The purpose of our study was to develop a pregnancy test that can fit all the ideal pregnancy test criteria. Those criteria are 1) high sensitivity (i.e., correct identification of pregnant animals); 2) high specificity (i.e., correctly identify non-pregnant animals); 3) inexpensive; 4) simple to conduct under farm condition; 5) have the ability to determine pregnancy status at the time the test is performed; and 6) have the ability to determine pregnancy status non-invasively.

Despite that hematocrit changes did not follow our hypothesis in this thesis, there were detectable differences in hematocrit levels, and assumed that plasma volume was also increased, between pregnant and non-pregnant sheep early in gestation. In the case of dairy cows, hematocrit levels were not consistent in their patterns. Perhaps hematocrit is not a valid marker for plasma volume. This means that developing a pregnancy test based on blood volume is still a valid idea. We have two main objectives in the future. First, conduct more research to understand

the changes in blood volume and components in different stages of pregnancy. Second, develop a less invasive method to measure blood volume.

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