

# REPRODUCTIVE ULTRASOUND FOR MANAGEMENT OF BEEF CATTLE

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## Introduction

O. J. Ginther stated: “gray-scale diagnostic ultrasonography is the most profound technological advance in the field of large animal research and clinical reproduction since the introduction of transrectal palpation and radioimmunoassay of circulating hormones.” (Ginther, 1986). It is hard to imagine that many discoveries and procedures related to ovarian, uterine and fetal function that we use today would have been considered without the development of real-time ultrasound. The research and commercial applications of ultrasound developed for reproduction over the last 15 years would support the statement by O. J. Ginther.

The area that has arguably benefited more from the development of ultrasound technology than any other area is reproduction in large animals. In many cases, rectal palpation has been replaced by transrectal ultrasonography for pregnancy determination, and diagnoses associated with uterine and ovarian infections. In addition, ultrasonography has added benefits such as fetal sexing, early embryonic detection and is less invasive than rectal palpation. From a research standpoint, ultrasound has given us the ability to visually characterize the uterus, fetus, ovary, corpus luteum, and follicles. More accurate measurements of the reproductive organs has opened doors to new areas of research and validated or refuted data from past reports.

Practical applications of ultrasound by bovine practitioners for routine reproductive examinations of beef and dairy cattle is the next contribution this technology is positioned to make to the livestock industry. Most veterinary students continue to be taught that ultrasound is a secondary technology for bovine reproductive work; however, the information-gathering capabilities of ultrasonic imaging far exceed those of rectal palpation. This paper will discuss the impact and practical applications of ultrasound for conducting routine reproductive examinations in dairy cattle.

## Veterinary Ultrasound Equipment

In general, linear-array, real-time, B-mode ultrasound scanners are best suited for veterinary applications involving cattle reproduction. Most ultrasound machines consist of a console unit that contains the electronics, controls, and a screen upon which the ultrasound image is visualized by the operator, and a transducer, which emits and receives high-frequency ultrasound waves. Linear-array transducers consist of a series of piezo electric crystals arranged in a row. These crystals emit high frequency sound waves upon being energized. The configuration of a linear-array transducer results in a rectangular image on the field of scan (as opposed to a pie shaped image produced by a sector transducer).

Bovine reproductive organs are most commonly scanned per rectum using a linear-array transducer specifically manufactured for transrectal use. However, specialized applications including ovum pickup and follicle ablation involve a transvaginal approach using a sector

transducer. Linear-array transducers of 5.0 and 7.5 MHz frequency ranges are most commonly used in cattle, and most veterinary ultrasound scanners are compatible with probes of different frequencies. Depth of tissue penetration of sound waves and image resolution is dependent upon and inversely related to the frequency of the transducer. Thus, a 5.0 MHz transducer results in greater tissue penetration and lesser image detail, whereas a 7.5 MHz transducer results in lesser tissue penetration and greater image detail. An ultrasound scanner equipped with a 5.0 MHz transducer is most useful for bovine practitioners conducting routine reproductive examinations, however, small ovarian structures such as developing follicles are best imaged with a 7.5 MHz transducer.

It is clear that ultrasound has made a tremendous impact as a scientific tool; however, ultrasound holds much promise as a tool to improve reproductive management in beef and dairy operations. There are several reasons that transrectal ultrasound is not widely used among bovine practitioners at present. First, research-grade ultrasound machines are relatively expensive, costing from \$10,000 to \$20,000. Second, most ultrasound machines require a cart and an external power source, thereby making them cumbersome to use in free-stall barns under field conditions. Recently, several ultrasound manufacturers have developed and marketed ultrasound machines that are cheaper, smaller, and battery operated. At present, these portable ultrasound machines lack the image quality of the larger console based units but may be easier to use on a routine basis. Continuation of the trend toward portability will foster future use of this technology by bovine practitioners for routine reproductive management.

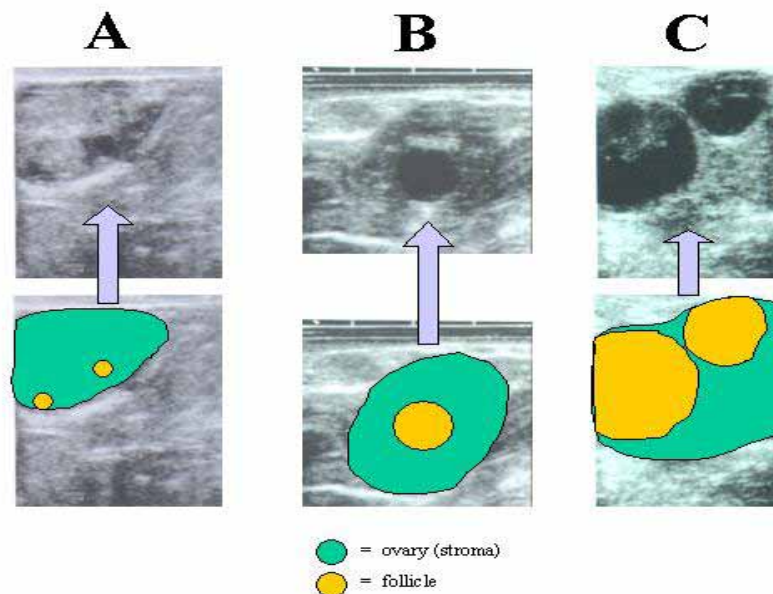
### **Imaging the Bovine Ovary**

Ovarian stroma, ovarian vessels, follicles, cysts, corpora haemorrhagica (CH), and corpora lutea (CL) are all structures that can be identified by real-time ultrasonography. The most distinguishable ovarian structures are antral follicles. Because follicles are fluid-filled structures they absorb ultrasound waves and are displayed as black on the screen (i.e., anechoic or non-echogenic). In contrast, the ovarian stroma, CH, and CL all contain varying degrees of dense cells, which reflect the ultrasound waves and result in a gray image on the screen.

Routine reproductive examinations should include visualization of the major structures (or the lack thereof) on both ovaries. Although rectal palpation can be an accurate method for diagnosing pregnancy, rectal palpation is a poor method for resolving ovarian follicles. By contrast, ultrasonic imaging is a highly accurate and rapid method for assessing ovarian structures. Too often, bovine practitioners proceed directly to scanning the uterus during reproductive examinations and neglect the ovaries all together. This is unfortunate because the ovaries contain a wealth of information that can be used to aid in diagnosing the reproductive status of the cow and for selecting appropriate therapies or reproductive interventions. For example, presence or absence of a corpus luteum aids in diagnosing pregnancy status, especially when conducting pregnancy exams early post-AI. When present, the size and location (i.e., left vs. right ovary) of the corpus luteum indicates the location of the conceptus within the uterus if the cow is pregnant. Ovarian pathologies such as “static ovaries” and follicular and luteinized cysts can easily be distinguished. Use of ovarian structures as diagnostic aids during reproductive examinations, however, requires a

thorough understanding of ovarian and reproductive anatomy and physiology. In addition, there are limitations to the conclusions that can be made from a single (as opposed to serial) ultrasound examination.

Manual palpation or ultrasonographic examination of the cow's genital tract are currently used by veterinarians involved in reproductive management. Ultrasound is effective at identifying follicles greater than 10 mm in diameter than rectal palpation. Follicles 10 to 15 mm in diameter were detected in 90% of cases using ultrasonography versus 62% of the cases using rectal palpation. Follicles greater than 15 mm were detected in 100% of the cases for both ultrasonography and rectal palpation. In a similar review manual diagnosis of follicles <10 mm was inaccurate, but ultrasound offered the possibility to diagnose follicles <5 mm and to measure the diameter of those follicles. Figure 1 demonstrates the appearance of the ovary at various stages of follicular development prior to emergence of a follicular wave, during proestrus, and after development of a follicular cyst.



**Figure 1.** Ultrasound image of bovine ovaries prior to emergence of a follicular wave (note two small follicles [ $< 5$  mm]; Panel A), during proestrus (note preovulatory follicle [13 mm]; Panel B), and after development of a follicular cyst (note delamination of granulosa layer into the antrum; Panel C). Images were taken using a 7.5 Mhz transducer (Lamb, 2001).

The CL is a transient endocrine gland that forms after ovulation from the tissues that previously composed the ovarian follicle. Thus, the CL can be viewed as the terminal stage of follicular development. Corpora lutea appear as distinctly echogenic areas within the

ovarian stroma. Many corpora lutea appear as a solid tissue masses but may also contain fluid-filled cavities. Based on ultrasonographic examinations in dairy heifers, 79% of otherwise normal CL contain cavities ranging from less than 2 to greater than 10 mm in diameter at some time during the estrous cycle and early pregnancy (Kastelic et al., 1990b). We (Spell et al., 2001) determined that luteal diameter was not associated with concentrations of progesterone on day 7 of the estrous cycle, but area and volume were correlated to concentrations of progesterone.

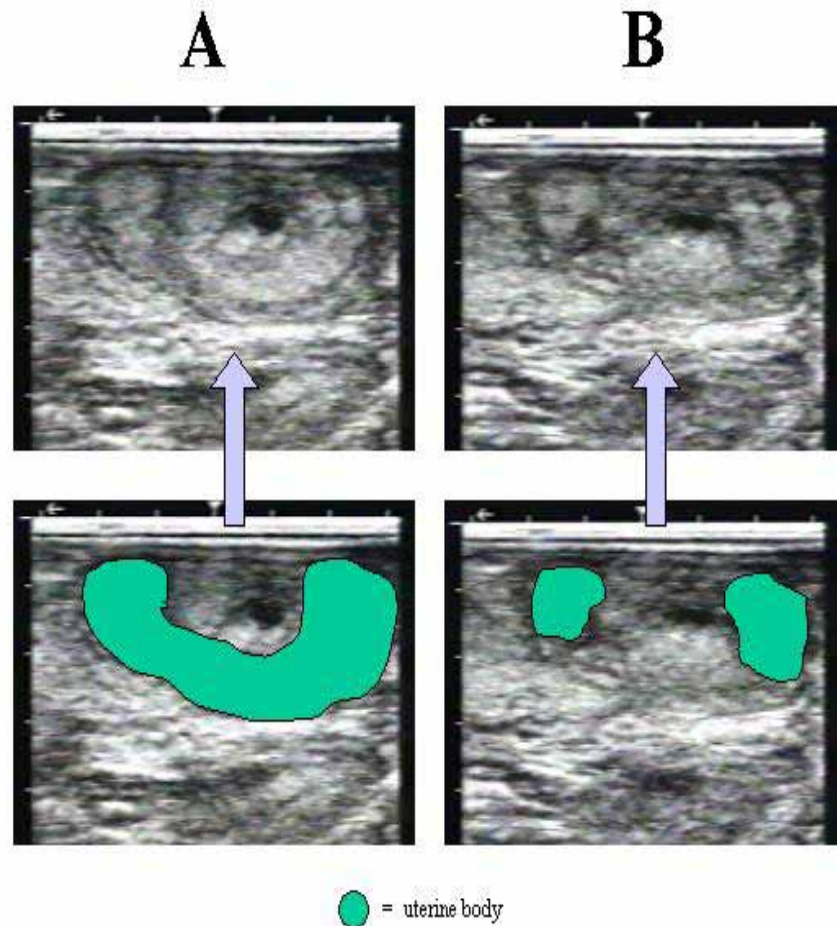
Ultrasonographic attributes of CL including cross-sectional diameter, luteal area, and echogenicity have been correlated to luteal structure and function (Battocchio et al., 1999; Kastelic et al., 1990a; Singh et al., 1997). Use of luteal characteristics to improve accuracy of pregnancy diagnosis has been reported in dairy heifers (Kastelic et al., 1991), but similar data does not exist for beef or lactating dairy cattle. Luteal size and echogenic characteristics assessed at specific times post breeding may prove useful as a method to improve accuracy of early pregnancy diagnosis in dairy cattle. Although ultrasound is more accurate than rectal palpation for assessing ovarian follicles, it is difficult to distinguish between developing corpora lutea and older regressing corpora lutea using either technique (Pieterse et al., 1990).

Under most circumstances, practical application of ultrasound for routine reproductive management consists of a single ultrasound examination at a given point in time. It is important to understand that the physiological status of a follicle (e.g., dominant, subordinate, growing, regressing) or corpus luteum cannot be determined during a single ultrasound exam. In addition, ultrasonic imaging aids in distinguishing anatomical attributes of a structure but confers little information regarding physiological or endocrine status. For example, ovarian cysts can be categorized by anatomical attributes such as diameter and presence or absence of luteal tissue; however, no information regarding functionality such as plasma hormone concentrations can be conferred. One exception would be the visualization of a fetal heartbeat as a diagnostic indicator of a viable fetus. The diagnostic limitation of ultrasonic imaging becomes important especially when the limitation is exceeded and an incorrect therapy or reproductive intervention is recommended. A thorough understanding of ovarian physiology and the mechanisms by which hormonal programs succeed or fail is imperative for correct interpretation of ultrasonic imaging information.

### **Imaging the Bovine Uterus and Conceptus**

Of all the ultrasound applications utilized by technicians in the industry, scanning of the uterus for infection and pregnancy are the most commonly practiced commercial applications that we have seen in the cattle industry. In a nonpregnant, cycling cow the uterine tissue appears as a somewhat echogenic structure on the screen. Because the uterus is comprised of soft tissue it absorbs a portion of the ultrasound waves and reflects a portion of the waves. In this way we can identify the uterus as a gray structure on the screen. A cross-sectional view of the uterus is displayed as a “rosette” and is easily distinguished from other peripheral tissues, whereas the longitudinal section is less recognizable, yet a trained technician can differentiate between the elongated view of the uterus and other tissues that may appear similar (Figure 2). Physiological changes during the estrous cycle leads to physical changes (such as tone) in the uterus, which alters the echogenic properties of the uterus (Pierson and Ginther, 1988). Even though a scoring system has been developed to

describe changes in uterine echogenic ability during different stages of the estrous cycle (Pierson and Ginther, 1988), predicting the stage of the estrous cycle remains inconsistent.



**Figure 2.** Ultrasound image depicting an elongated (Panel A) and cross-sectional (Panel B) view of the nonpregnant uterus. Images were taken using a 5.0 Mhz transducer (Lamb, 2001).

Pathological applications for ultrasound technology have extended to identifying endometritis, pyometra, mucometra, and hydrometra. With the aid of ultrasound, researchers have determined that uterine infections were related to delayed postpartum folliculogenesis, to the occurrence of short luteal phases after the first postpartum ovulation, and to the development of follicular cysts on the ovaries.

Detection of the embryo proper as well as embryonic and fetal developmental characteristics during early fetal development are shown in Table 2. The bovine fetus can be visualized beginning at 20 d post breeding and continuing throughout gestation, however,

because of its size in relation to the image field of view, the fetus cannot be imaged *in toto* after about 90 days using a 5.0 MHz linear-array transducer.

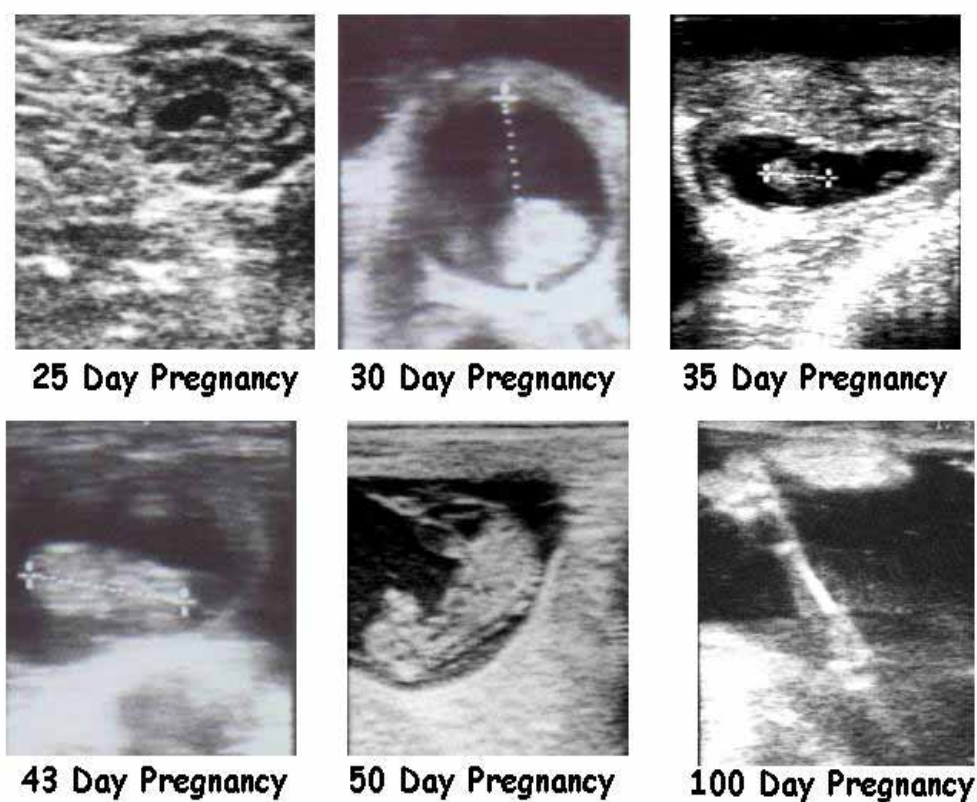
**Table 2.** Day of first detection of ultrasonographically identifiable characteristics of the bovine conceptus (Adapted from Curran et al., 1986).

Characteristic	First day detected	
	Mean	Range
Embryo proper	20.3	19 to 24
Heartbeat	20.9	19 to 24
Allantois	23.2	22 to 25
Spinal cord	29.1	26 to 33
Forelimb buds	29.1	28 to 31
Anmion	29.5	28 to 33
Eye orbit	30.2	29 to 33
Hindlimb buds	31.2	30 to 33
Placentomes	35.2	33 to 38
Split hooves	44.6	42 to 49
Fetal movement	44.8	42 to 50
Ribs	52.8	51 to 55

### *Early Pregnancy Diagnosis*

Reports have indicated the detection of an embryonic vesicle in cattle as early as 9 of gestation. In these situations the exact date of insemination was known and ultrasonography simply was used as a confirmation of pregnancy or to validate that detection of an embryo was possible within the first two weeks of pregnancy. In contrast, Kastelic et al. (1989) monitored pregnancy in pregnant and nonpregnant yearling heifers that were all inseminated. Diagnosis of pregnancy in heifers on day 10 through day 16 of gestation resulted in a positive diagnosis for pregnant or nonpregnant of less than 50%. On days 18, 20, and 22 of gestation accuracy of pregnancy diagnosis improved to 85%, 100%, and 100%, respectively. Although evidence of a pregnancy via ultrasound during days 18 to 22 of gestation yields excellent results, a technician needs to ensure that confusion between fluid accumulation in the chorioallantois during early pregnancy and uterine fluid within the uterus during proestrus and estrus are not confused when making the diagnosis.

Although we have indicated that an embryonic vesicle is detectable by ultrasound as early as 9 days of gestation, accuracy of detection approaches 100% after day 25 of gestation. For practical purposes, the efficiency (i.e., speed and accuracy) of a correct diagnosis of pregnancy should be performed in females expected to have embryos that are at least 26 days of age (Figure 3). This information can be used to determine the age of bovine fetuses with a high degree of accuracy. Crown-Rump length measurements were summarized by Hughes and Davies (1989; Table 3). There was a significant correlation ( $r = 0.98$ ) between embryo age and crown-rump length.



**Figure 3.** Ultrasound images of the bovine fetus at various stages of development

**Table 3.** Fetal crown-rump length in relation to age in weeks (Hughes and Davies, 1989).

Fetal age, weeks	No. of observations	Crown-rump length, mm		
		Minimum	Maximum	Mean
4	25	6	11	8.9
5	35	8	19	12.8
6	50	16	26	20.2
7	47	23	36	27.7
8	41	36	52	45.5
9	48	39	71	62.4
10	43	61	101	87.4
11	39	95	118	106.5
12	32	107	137	121.8

Ultrasound is a rapid method for pregnancy diagnosis, and experienced palpators adapt to ultrasound quickly. The time required to assess pregnancy in beef heifers at the end of a

108-day breeding season averaged 11.3 seconds using palpation per rectum versus 16.1 seconds required to assess pregnancy and fetal age using ultrasound (Galland et al., 1994). Fetal age also affected time required for diagnosis with older fetuses requiring less total time for diagnosis. Although ultrasound at  $\geq 45$  d of gestation did not increase accuracy of pregnancy diagnosis for an experienced palpator, it may improve diagnostic accuracy of a less experienced one.

Two caveats must be considered when using ultrasound for routine early pregnancy diagnosis in a cowherd. First, when using ultrasound for early pregnancy diagnosis, emphasis must be given to identifying nonpregnant rather than pregnant cows. Second, a management strategy must be implemented to return the nonpregnant cows to service as quickly as possible after pregnancy diagnosis. Such strategies include administration of PGF<sub>2 $\alpha$</sub>  to cows with a responsive CL, use of estrus detection aids, or a combination of both methods.

### **Early Embryonic Loss**

Prior to the development of ultrasound for pregnancy diagnosis in cattle, technicians were unable to accurately determine the viability or number of embryos or fetuses. Because the heartbeat of a fetus can be detected at approximately 22 days of age, we can accurately assess whether or not the pregnancy is viable. Studies in beef and dairy cattle have used ultrasound to assess the incidence of embryonic loss. The number of fetuses can most accurately be assessed at between 49 and 55 days of gestation (Davis and Haibel, 1993).

**Table 4.** Incidence of embryonic/fetal loss in cows after an initial diagnosis of pregnancy by ultrasound, followed by a second diagnosis prior to or at calving

Reference	No. pregnant, days of gestation	No. pregnant, days of gestation	No. of embryos lost	Embryonic mortality, %
<b>Beef Cattle</b>				
Beal et al., 1992 (Cows)	138	129	9	6.5
	25 days	45 days		
	129	127	2	1.5
	45 days	65 days		
	138	127	11	8.0
	25 days	65 days		
Lamb et al., 1997 (Heifers)	149	143	6	4.0
	30 days	60 days		
	271	260	11	4.1
	35 days	75 days		
	105	100	5	4.8
	30 days	90 days		

Table 4 summarizes the incidence of embryonic loss by study in beef and dairy females. The fertilization rate after artificial insemination in beef cows is 90%, whereas embryonic survival rate is 93% by day 8 and only 56% by day 12 post artificial insemination (Diskin and Sreenan, 1980). The incidence of embryonic loss in beef cattle appears to be significantly less than in dairy cattle. Beal et al. (1992) reports a 6.5% incidence of

embryonic loss in beef cows from day 25 of gestation to day 45. Similarly, Lamb et al. (1997) noted a 4.2% incidence of embryonic loss in beef heifers initially ultrasounded at day 30 of gestation and subsequently palpated rectally at between day 60 and 90 after insemination. These studies indicate the usefulness of ultrasonography as a tool to monitor the success of a breeding program, by determining pregnancy rates and embryonic death.

At present, there is no practical way to reduce early embryonic loss in lactating dairy cows. However, recognizing the occurrence and magnitude of early embryonic loss may actually present management opportunities by taking advantage of new reproductive technologies that increase AI service rate in a dairy herd. If used routinely, transrectal ultrasonography has the potential to improve reproductive efficiency within a herd by reducing the period from AI to pregnancy diagnosis to 26 to 28 days with a high degree of diagnostic accuracy.

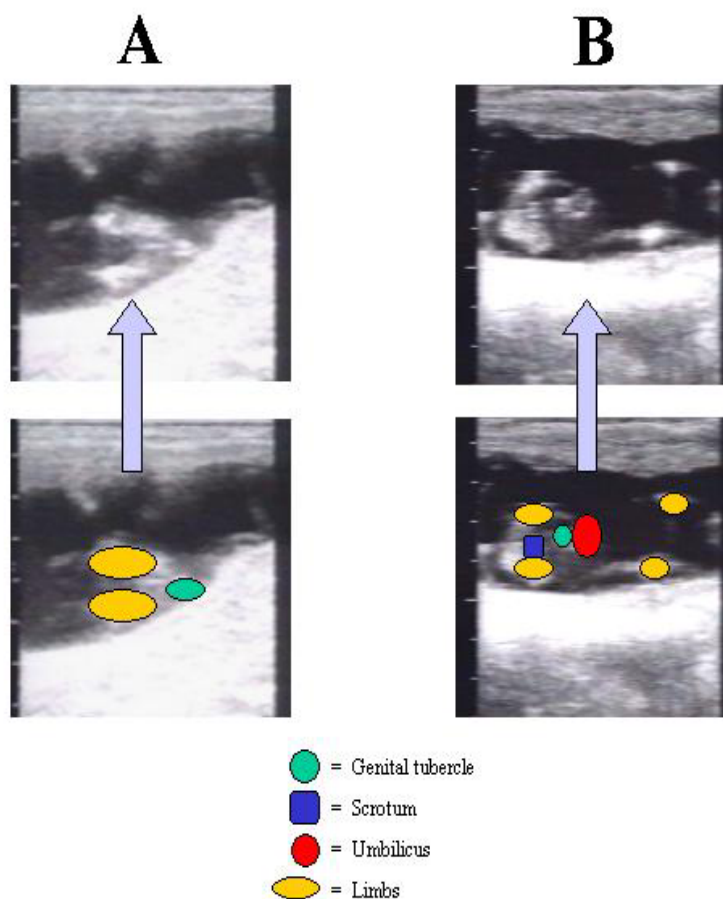
### **Fetal Sexing**

Many cattle operations are developing strategies to use fetal sexing as either a marketing or purchasing tool. At approximately day 50 of gestation, male and female fetuses can be differentiated by the relative location of the genital tubercle and development of the genital swellings into the scrotum in male fetuses. Fetuses at 48 to 119 days of age have been successfully sexed. The procedure is reliable and accuracy has ranged from 92 to 100%. Beal et al. (1992) noted that of 85 fetuses predicted to be male 84 were confirmed correct, resulting in 99% accuracy. In addition, of 101 fetuses predicted to be female 98 were confirmed correct, resulting in 97% accuracy. Recently, we (Lamb, 2001) determined the sex of 112 fetuses in Angus heifers with 100% accuracy.

For optimal results the ultrasound transducer should be manipulated to produce a frontal, cross-sectional, or sagittal image of the ventral body surface of the fetus. In larger framed cows (i.e. Holsteins and Continental beef breeds) or older cows the optimum window for fetal sexing usually is between day 55 and 70 of gestation, whereas for smaller framed cows (Jerseys and English beef breeds) the ideal window usually is between day 55 and 80 of gestation. There are two limitations that could inhibit the ability of a technician to determine the sex of a fetus: 1) as the fetus increases in size it becomes more difficult to move the transducer relative to the fetus to obtain the desired image; and, 2) the gravid horn is more likely to descend ventrally into the abdominal cavity in larger or older cows, making fetal sexing virtually impossible without retracting the gravid horn.

Figure 4 illustrates the cross-sectional image of female fetus (65 days of gestation; Panel A) and a sagittal view of a male fetus (65 days of gestation; Panel B; Lamb, 2001). The umbilicus can be used as an excellent landmark when determining the location of the genital tubercle or presence of a scrotum in males. In the male, the genital tubercle is located adjacent to and caudal to the umbilicus, whereas the genital tubercle in the female is located just ventral to the tail. The scrotum is detectable between the hind legs of the male fetus. The genital tubercle and scrotum are echogenic and are easily detected on an ultrasound screen as echogenic images. To ensure an accurate diagnosis of sex, for each patient, a technician should view an image at three locations: 1) adjacent to the umbilicus, where the umbilicus enters the abdomen (possible male genital tubercle); 2) the area between the back legs (possible scrotum); and, 3) ventral to the tail (possible female genital tubercle).

In beef cattle operations, fetal sexing remains limited to purebred operations especially in conjunction with an embryo transfer program. Determination of sex especially after the successful transfer of embryos to recipients allows marketing of male and female embryos before the pregnancy is carried to term. This strategy can be used effectively in dairy operations trying to produce bull calves of a particular mating for sale to bull studs. From a commercial cattle operation standpoint, heifer development operations are utilizing fetal sexing as a marketing tool to provide potential buyers with females that are pregnant with fetuses of a specific sex. As more technicians become proficient at fetal sexing, commercial operations will utilize this technology to enhance the marketability and efficiency of their cattle operations.



**Figure 4.** Ultrasound image of a female bovine fetus (65 days of gestation; Panel A) and a sagittal view of a male fetus (65 days of gestation; Panel B). Images were taken using a 5.0 Mhz transducer.

### Conclusion

The impact of real-time ultrasound on the study of reproduction has been dramatic and the further development of portable ultrasound machines has given clinicians an added tool for diagnostic reproductive management. Ultrasound is commonly used to monitor uterine anatomy, involution, and pathology. In addition, it has been used to detect pregnancy, study embryonic mortality, monitor fetal development, and determine fetal sex. The applications of ultrasound used by scientists include the ability to monitor follicular characteristics,

ovarian function, and aid in follicular aspirations and oocyte retrieval. In the future, as technology improves technicians will have an opportunity to use the internet or video conferencing for ultrasound image analyses. With every new technological development, scientists, veterinarians, and producers discover new possibilities for the use of reproductive ultrasound to enhance the scientific merit of research or improve reproductive efficiency in cattle operations.

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# SOMATIC CELL CLONING IN THE BEEF INDUSTRY

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## INTRODUCTION

Technically, cloning is the production of multiple genetically identical animals. Surprisingly, genetically identical animals have been produced for over 30 years using a technique of dividing embryos into two or more portions to produce multiple embryos. This technique was termed “splitting” and consequently did not generate as much public interest, or concern, as the current technique of nuclear transplantation which, for better or worse, is commonly referred to as “cloning”. Although splitting has been done commercially for many years its application is limited by the fact that only embryos can be divided and only two copies can be made. With each division of the embryo the resulting portions become smaller and less viable. Nuclear transplantation is an entirely different approach and has the potential of producing an unlimited number of genetically identical animals (Chan, 1999; Cibelli et al., 1998; Kato et al., 1998; Polejaeva and Campbell, 2000; Stice et al., 1998; Zakhartchenko et al., 1999a; Wilmut et al., 1997).

The theoretical basis for using nuclear transplantation for cloning is that all nuclei from an organism, with only a few exceptions, contain the exact same set of genes (Kato et al., 1998, 1999; Stice et al., 1998; Zakhartchenko et al., 1999a,b; Wakayama et al 1998, 1999; Wilmut et al., 1997). A skin cell contains the same set of genes as a liver cell, a kidney cell or any other cell in the body. Furthermore, small cell samples can be taken from an individual and greatly expanded in culture. Therefore, many genetic copies of an individual are technically available for making clones.

In each of these cells, however, a specific set of genes is used which results in each cell type having a unique function. We, therefore, need a method of turning off the genes that these cells are using in their original form and turning on the genes appropriate for each cell type in a complete, fully formed individual (Stice et al., 1998). The embryo does this naturally so the objective is to turn the cell into an embryo. The simple-minded approach that has been used is the removal of the genetic material from an egg and its replacement with the genetic material from one of the millions of somatic, or body cells, from the individual to be cloned. And, believe it or not, this actually works.

Techniques for the genetic modification of animals have also been available since the early 1980's (Chan, 1999; Piedrahita et al., 1999). As with cloning great interest was generated for application to farm animals; especially following a report of genetically modified mice that grew to 40% larger than nontransgenic littermates. The method of making genetic modifications in animals at this time was to microinject a few hundred to a few thousand copies of a gene into the pronucleus of a newly fertilized embryo (Chan, 1999; Piedrahita et al., 1999). As with cloning this method was used to produce a few transgenic cattle, sheep, goats and pigs. However, the method was limited, as for cloning, by the availability and expense of embryos and recipients. The nuclear transplantation method using cultured cells for nuclear donors has provided a much simpler and

more efficient method for genetically modifying agricultural animals (Chan, 1999; Cibelli et al., 1998; Piedrahita et al., 1999; Polejaeva and Campbell, 2000; Stice et al., 1998; Zakhartchenko et al., 1999a). Also, for the first time, it is now possible to insert a length of DNA into a predetermined site in the genome of agricultural animals (Chan, 1999; Cibelli et al., 1998; Piedrahita et al., 1999; Polejaeva and Campbell, 2000; Stice et al., 1998; Zakhartchenko et al., 1999a).

## **HISTORY OF MAMMALIAN CLONING RESEARCH**

The method of nuclear transplantation was first developed and used successfully in amphibians in the 1960's (Wilson et al., 1995). Although several attempts were made to develop an efficient procedure for use in mammals over the years, it wasn't until 1983 that a technique was developed in mice that led to the first work in agricultural species (Wilson et al., 1995). This method is elegant and highly efficient.

The method first involves the removal of the genetic material from a recipient cell using a unique noninvasive approach. Although a sharpened micropipet is used for the procedure the cell membrane is so resilient that it is not penetrated (Robl et al., 1987). The pipet can be moved adjacent to the genetic material and with aspiration the genetic material is sucked into a membrane-bounded pocket, which is then pinched off from the cell. The membranes seal and the cell is perfectly intact.

Completion of the nuclear transplant process required the development of a method for inserting a nucleus into the enucleated recipient cytoplasm. To avoid disrupting the recipient cell membrane methods of cell fusion were attempted and proved to be successful (Robl et al., 1987). The resulting method for transplanting nuclei from one cell to another in mice was efficient and provided hope that similar methods could be practical for agricultural species.

Work began immediately to develop nuclear transplantation procedures for cows, pigs and sheep. This work was particularly difficult because efficient in vitro culture methods for oocytes and embryos had not yet been developed for these species. Ovulated oocytes and embryos, recovered from superovulated animals sent to slaughter were used. Resulting nuclear transplant embryos were transferred to the oviducts of sheep for several days before being recovered and transferred into recipient cows as blastocyst stage embryos (Robl et al., 1987).

Furthermore, methods developed for the mouse were not directly transferable to cows, sheep and pigs. The Sendai virus fusion system did not work at all. The cytoplasm of embryos in these species is much more opaque than the mouse making enucleation techniques more difficult and the size of the cells is different.

However, one of the greatest challenges in cloning embryos from agricultural species was not related to these technical difficulties. Interestingly, not long after the highly efficient procedure for nuclear transplantation in mice had been published a second paper was published by the same group of investigators reporting that the procedure would not work for cloning and, in fact, that "cloning by simple nuclear transplantation was biologically impossible". The mouse proved to be a very poor model for cloning and real success with this species was not realized until recently.

Fortunately, work persisted on cloning agricultural species and the first success was reported in the sheep in 1986, in the cow in 1987 and in the pig in 1989 (Robl et al., 1987).

Though successful, all cloning work at this time was based on a fallacy. Prior work in amphibians, and work that still stands today, indicated that cloning success decreased as a cell differentiation increased. Differentiation is the specialization that occurs in cells as the various organs and tissues form during embryo development. The thought was that differentiation was the result of a specific set of genes being turned on and the coincident inactivation of many other genes not needed in that specific cell type. It was thought that the inactivation of genes was an irreversible event, therefore, differentiation resulted in a cell having a specific function that could not be changed.

Consequently, cloning was done in agricultural animals, up until recently, using donor cells from early embryos (Robl et al., 1987). These cells were thought to be less differentiated than cells from either fetuses or adult animals. One of the disadvantages of using early embryos is that the number of nuclear donor cells is limited so the number of clones that can be produced is limited and efficiency is of utmost importance. For example, if a 32-cell embryo is used and the efficiency is 25% blastocyst production and 25% of these embryos surviving to term then the average number of clones produced is 2; the same as with embryo splitting.

During the decade following the first successes with cloning tremendous effort was placed on improving the efficiency of the embryo cell cloning procedure. One aspect of this effort was to develop and improve in vitro oocyte maturation and embryo culture systems in the cow and pig. Success was first realized in the cow, which greatly facilitated further research by making cow gametes and embryos readily available. A second aspect was to develop a better understanding of early development in agricultural species. Prior to this time nearly all work on mammalian development had been done in the mouse and very little was known about the normal process of embryo development in cows and pigs. This information was necessary for designing cloning manipulations that would simulate normal development as closely as possible. The third aspect of improving efficiency was developing novel approaches for manipulating embryos and refining these new methodologies. Ultimately, the embryo cell cloning procedure was proven not to be practical and interest in nuclear transplantation waned.

In 1997 the surprising and revolutionary discovery was made that differentiated cells could support development to term following fusion with an enucleated egg (Wilmut et al., 1997). This first work was done in sheep and was verified the following year in the cow. In the three years since this discovery was made interest in cloning research has exploded. To date somatic cell cloning has been successful in sheep, cow, goat, mouse and pig. Hundreds of cloned cattle and mice have been produced. Still work remains on understanding the process and improving efficiency.

### **NUCLEAR TRANSPLANTATION METHOD FOR CLONING CATTLE**

The nuclear transplantation method for cloning cattle begins with recovery and preparation of the donor cells from the animal to be cloned. Tissue is recovered as a small disc of skin from the back of the ear. The ear is shaved and washed with a disinfectant solution. A trochar is used to cut the disc of skin which is removed with a forceps, dipped in 70% ethanol placed in a tube containing a sterile saline solution (Standard Operating Procedures Of Cyagra). Good samples have been recovered from animals of any age and in some cases animals that have been dead for several hours (Cyagra of Kansas). The sample is then shipped to the laboratory by overnight express mail.

At the laboratory the skin sample is washed, cut into small pieces and digested with enzymes to free the fibroblast cells from the tissue. Interestingly, this process is not necessary and fibroblasts will actually creep out of the edges of the tissue, attach to the bottom of the culture dish and begin growing without any processing of the tissue at all. Generally, the cells are grown for several weeks, removed from the bottom of the dish with an enzyme treatment and then cryopreserved. Several days prior to nuclear transplantation a vial of the cells is thawed and grown until the cells cover the bottom of the dish (a confluent layer). The cells are then passed into multiple dishes to be used for several days of nuclear transplantation.

Oocytes are recovered from the ovaries of slaughtered animals. Ovaries are collected at the slaughterhouse then transported to the laboratory at room temperature. The oocytes are collected by aspirating antral follicles using a needle attached to a vacuum pump. The oocytes, in follicular fluid, are then sorted under a dissecting microscope and only oocytes with several layers of intact cumulus cells (the cells surrounding the egg) and a uniform cytoplasm are retained for use the following day as nuclear recipients.

Oocytes collected directly from ovarian follicles are not ready to be used in nuclear transplantation. They must undergo a process called maturation, which takes about 18 hours. After maturation the cumulus cells are removed from around the oocytes by shaking the cells vigorously in an enzyme solution. The mature oocyte is arrested, with condensed chromosomes, in the second metaphase of meiosis. It has extruded half of its DNA in a small cell called a polar body. The chromosomes are not readily visible in the oocyte and must be labeled with a fluorescent dye for removal.

Chromosome removal is done under a microscope at 400X magnification because the egg is only about 0.1 mm in diameter, about the size of the smallest speck of dust that can be seen. The microscope is set up with micromanipulators. The micromanipulators translate the very coarse movement of the hand into a very fine movement of a set of tiny glass micropipets. The micropipets are used to hold the egg and to remove the chromosomes. Chromosome removal involves fixing the egg in place with the holding pipet and inserting the enucleation pipet through the zona pellucida, the soft shell surrounding the egg. The chromosomes are located by a flash of UV light, which makes the fluorescent dye visible. The enucleation pipet is moved adjacent to the chromosomes and with aspiration are removed in a membrane enclosed bleb.

Donor cells are simply removed from the dishes using an enzyme treatment. They are then picked up individually and placed between the zona pellucida and the egg. These couplets are then fused together using a high voltage electrical shock. The electrical pulse creates a charge across the cell membranes pulling the nuclear donor cell tightly against the recipient cytoplasm. At some point the charge becomes sufficiently high that it ruptures the membranes creating many tiny holes between the two cells. When the holes reseal the two cells fuse together.

The next step in the process involves stimulating the nuclear transfer embryo to divide. Normally when the egg is fertilized the sperm imparts a signal that initiates cell division. Because we are omitting the sperm in nuclear transfer we have to use an artificial stimulus to initiate cell division. There are many approaches to doing this. The procedure we use consists of treating the nuclear transfer embryos with a chemical that elevates intracellular calcium following by a chemical that inhibits protein synthesis. Egg activation is a complex process and is currently an important area of further research.

The nuclear transplant embryos are then placed in culture for 7 to 9 days. During this time cell division takes place and some differentiation occurs so that the embryos have about 80 to 200 cells and cells that will form the placenta and fetus are clearly distinguishable. Then embryos are then transplanted into recipients for development to term.

## **RESULTS OF SOMATIC CELL CLONING IN CATTLE**

To date, there are about 150 calves that have been produced using somatic cell nuclear transplantation in the world. About 30 different laboratories, with most being in Japan, have produced these calves. Overall development rate from nuclear transfer to healthy calf at term is about 1 to 3%.

A more detailed analysis provides information on when losses occur. The results can vary greatly depending on many factors; some known and many unknown. In general, development of cloned embryos in culture to the blastocyst stage at day 7 to 9 is about 15 to 20% but can vary from 5 to 50%. Pregnancy rates, transferring either one or two embryos per recipient, are generally high (40 to 60%) at day 35 but substantial losses occur by day 60 to 90 resulting in pregnancy rates of about 25% (Cyagra Data). Although some losses occur during the middle trimester of gestation they are generally low. In the last trimester substantial losses occur resulting in about 10 to 20% of the calves surviving to term (Hill et al., 1999). Unfortunately, perinatal and postnatal losses can be as high as 50% but with intensive care can be reduced to about 10 to 20%.

Prenatal, perinatal and postnatal losses have been documented in some detail. Prenatal losses generally occur as a result of fluid accumulation in the placenta (hydrops). The fluid accumulation gets sufficiently high that the cow aborts the calf. On gross examination the calf may look normal. Most calves have been taken by Caesarian section. Hydrops is noted in many of these pregnancies (Hill et al., 1999). In addition, the calves have an extraordinarily large umbilicus (Hill et al., 1999). They also typically have breathing difficulties and benefit from being placed on oxygen for a few hours (Hill et al., 1999). They also may have a variety of other problems such as fluid accumulation in various organs. By the first few days after birth most calves appear normal but may exhibit digestive problems. By sixty days of age the calves have generally outgrown any defects they might have had at birth.

The defects that are seen are remarkably consistent from lab to lab and appear to stem from gestational problems as opposed to genetic defects. Analysis of early conceptuses indicates that the placenta is likely the cause of most of the problems that have been observed. For unknown reasons placental attachment to the uterine lining does not occur properly in cloned embryos. This lack of adequate attachment may result in the fluid accumulation, large umbilical cords and fluid accumulation in various organs that has been observed. The defect is sometimes observed in calves derived from in vitro produced embryos, which indicates that the defect may be the result of poor culture systems. However, it is unlikely that in vitro culture is the only cause of the problem (Cibelli et al., 1998).

## **CLONING IN THE CATTLE INDUSTRY**

Somatic cell cloning has great promise but the limitations of low pregnancy rates and calf survival restrict its current use. At this time somatic cell cloning is still in the research phase. Considerable work needs to be done to improve survival rates and evaluate variations in results before cloning can be commercialized. The second phase will be small-scale commercialization of the technology to multiply animals of high value. As efficiency and the quality of embryos improves, cryopreservation will become feasible and large numbers of embryos will be sold in straws just as semen is today.

The ability to clone adult animals from easily cultured skin cells presents several opportunities to the beef industry. One opportunity is to preserve valued genotypes. To maintain the genetics of any animal in the herd indefinitely, a skin sample can be taken, processed and the cells frozen back for future use. Unlike semen, the cells contain a full copy of the genetic material rather than just half the chromosomes. Unlike embryos, the phenotype of the animal is known.

Cloning may also present a unique opportunity for marketing genetics. Evaluation of an animal by performance and progeny testing is costly and takes several years. Cloning may be useful in increasing the return from fully tested animals. These animals would be of great value to producers because of the predictability of the outcome.

Cloning could facilitate integration of segments of the beef industry for the production of specialty brand name products. Highly uniform brand name products would be helpful in increasing both value and consumption of beef products. Examples of possible products are high lean, low fat beef from double muscled animals or high quality beef from Japanese Waygu animals. Increased uniformity of beef products will improve consumer appeal and reduce the cost of production.

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