

Bovine Bodyworks: Ultrasound Imaging Of Reproductive Events in Cows

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■ Take Home Messages

- Use of ultrasonography in bovine practice began in the 1980's
- Detectable changes in the uterus are predictive of the estrous cycle
- Ultrasonography is particularly useful for early pregnancy diagnosis, assessing fetal health, and fetal sex diagnosis
- Ultrasonography is not associated with higher embryonic loss
- Imaging the ovaries in cattle has led to an understanding of follicular wave dynamics in this and many other species, and to new protocols for ovarian synchronization and superstimulation
- Three-dimensional ultrasonography, ultrasound biomicroscopy, and computer-assisted analysis of ultrasound images will ultimately enable us to determine the precise stage of the estrous cycle based on a single examination, and the health status of individual follicles and their contained egg

■ Introduction

Since the 1600's, studies of reproductive function have involved data gathered from gross and microscopic examination of excised tissues. Even the most detailed studies of static specimens excluded the dynamic processes of life and the mechanisms controlling them. Although knowledge gained from the study of tissue samples has been considerable, understanding of structure-function relationships of the reproductive system was hindered until the 1980's when real-time, B-mode ultrasonography became available. The ability to visualize changes in structure in a serial fashion, without interruption or distortion of function, has revitalized the study of reproduction in many species, but most notably in cattle.

The following is an overview of ultrasound imaging techniques that have been used in the study of bovine reproduction during the last decades and of emerging techniques that hold promise in the future.

■ **Imaging the Uterus**

Characteristic changes of the tubular genitalia visible by ultrasonography involve thickness of the uterine body, evidence of increased vascularity and edema, and accumulations of mucus (Figure. 1; Pierson and Ginther, 1987; 1988). The period of proestrus and estrous Days -4 to -1 (Day 0 = ovulation) is characterized by 1) increasing thickness of the uterine body, 2) accumulation of luminal fluid first in the uterus followed in succession by fluid in the cervix and vagina, and 3) minimal curl of the uterine horns. Conversely, diestrus, (Days 3-16) is characterized by minimal thickness, minimal luminal fluid, and maximal curl to the uterine horns (Figure 1). Heterogeneous endometrial echotexture (interspersed areas of dark and bright gray) is reflective of uterine edema and associated with impending estrus and ovulation.

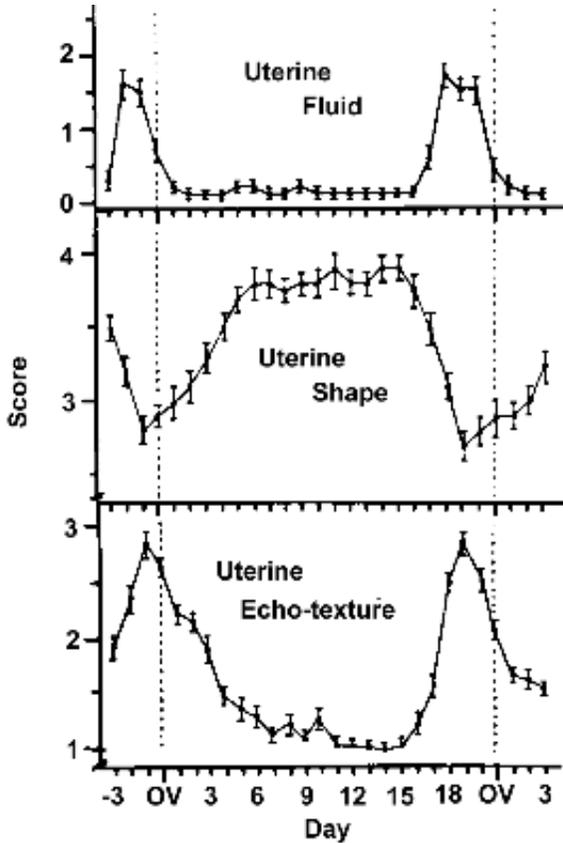


Figure 1. Changes in uterine luminal fluid (0, minimal; 3, maximal), degree of uterine horn curl (1, minimal; 4, maximal), and uterine echotexture (1, homogeneous; 3, heterogenous) during the estrous cycle in cattle (n=58; Adapted from Pierson & Ginther, 1987).

■ Imaging the Conceptus

Early Pregnancy Diagnosis

There are numerous reports of the use of ultrasonography for evaluating pregnancy in cattle (Griffin, 1992; Mee et al., 1994). As in the mare, early diagnosis of pregnancy is based on the detection of discrete, nonechogenic fluid pockets or lines within the uterine lumen, but must be subsequently confirmed by progressive elongation of the fluid pocket and detection of an embryo proper. Initial reports suggested that a presumptive diagnosis of pregnancy may be attempted as early as Day 10 by the detection of intraluminal fluid (see Kastelic et al., 1991). However, results of a later and very detailed study indicated that early diagnosis based solely on the

detection of fluid within the uterine lumen was unreliable because 1) the small dimensions of the elongating conceptus approach the limits of resolution of most available scanners (1 to 3 mm), and 2) small amounts of apparently normal free intrauterine fluid are present as early as Day 10 in nonbred cows which are indistinguishable from the fluid within the embryonic vesicle of pregnant cows (Kastelic et al., 1991). The accuracy of early pregnancy diagnosis was not greater than a guess (50%) before Day 18 using a 5 MHz transducer, or before Day 16 using a 7.5 MHz transducer. The accuracy of early pregnancy diagnosis based on fluid alone approached 100% by Day 20 (Kastelic et al., 1991). The ability to diagnose pregnancy at ≤ 21 days may be of great importance for progressive breeders who recognize the economic advantage of detecting nonpregnant cows before another estrus has passed. Characteristics inconsistent with a diagnosis of pregnancy at Day 20 are signs of impending estrus and ovulation, including intrauterine fluid that accumulates, on average, 2 or 3 days earlier than that of pregnancy, intravaginal fluid, increasing uterine tone, estrus-like uterine echotexture, and a regressing corpus luteum.

Regarding the expediency of the technique, results of one study (Galland et al., 1994) showed that an experienced operator can diagnose pregnancy and estimate fetal age (between 45 and 108 days) in an average of 16.1 seconds using ultrasonography, compared to 11.3 seconds for palpation. The authors concluded that use of ultrasound at ≥ 45 days of gestation may not increase the accuracy of diagnosis for an experienced palpator, but will for a less experienced one. More time was required to estimate fetal age in pregnancies between 65 and 85 days than those at either 45 - 60 days or 90 -108 days (Galland et al., 1994). The requirement for more time during the middle stage coincides with a period of relatively subtle changes in the fetus compared to other stages.

Does Ultrasonography Lead To More Embryonic Loss?

The use of ultrasonography to diagnose pregnancy is relatively simple, but does require some expertise to avoid incorrect diagnoses. When the uterus is located cranial to the pelvic inlet, a false negative diagnosis is more likely to be given than when the uterus is within the pelvic cavity (Szenci et al., 1995). Since diagnosis of pregnancy can be made earlier using ultrasonography than by palpation, it is possible that more pregnancy losses (early embryonic death) may be detected. However, the technique *per se* does not appear to cause embryonic death (Ball and Logue, 1994; Baxter and Ward, 1997). Furthermore, the incidence of pregnancy loss (cows that aborted or returned to estrus before term) after diagnosis was not different between cows that were 30, 40 or over 50 days pregnant when the diagnosis was performed (Baxter and Ward, 1997). Similar rates of pregnancy loss have been reported among studies involving transrectal ultrasonographic diagnosis of pregnancy in cattle: 5.5% loss after ultrasound diagnosis on days 30 to 40 (Baxter and

Ward, 1997), 8.6% after diagnosis on days 26 to 58 (Szenci et al., 1998), and an estimated 10% after diagnosis on days 25 to 45 (Mee et al., 1994). By comparison, in a study of 10 dairy herds involving 4,208 pregnancies (Forar et al., 1996), pregnancy loss (return to estrus, observed abortion, follow-up examination) following transrectal palpation at day 45 (median; range 38 to 80) of pregnancy was 10.8% (range among herds, 7.6 to 13.0%). The nature of embryonic loss has also been investigated in cattle, and ultrasonographic characteristics have been described (Kastelic, 1989, Baxter and Ward, 1997). Results of these studies have dispelled the mistaken notion that uterine "resorption" is the common mode of eliminating the early conceptus. Results indicate that embryo/fetal fluids and tissues are retained until the ensuing estrus when they are expelled through the cervix, usually unnoticed.

Embryo / Fetal Development

Detection of the embryo proper, embryonic heartbeat, and developmental characteristics of the bovine conceptus throughout the embryonic and fetal stages have been reported (Table 1, Curran et al., 1986; Kahn 1989). During early pregnancy, the uterine horns in pregnant cattle become compartmentalized by segmental contraction of circular smooth muscle, and it was concluded that formation of several chambers is the reason the bovine embryonic vesicle cannot be imaged *in toto* (Kahn et al., 1989). The bovine fetus can be imaged throughout gestation, but becomes too large to image in its entirety using a standard 5 MHz probe beyond about 90 days. After about 7 months of gestation, the size and depth of the uterus may result in failure to image the fetus without the use of a lower frequency probe that provides deeper tissue penetration (e.g., 3.5 MHz). Ultrasonography has also been used to accurately assess the incidence of multiple fetuses in cattle, but only after the moment when the embryo proper becomes detectable (i.e., ≥ 20 days). Multiple fetuses were accurately diagnosed between 49 and 55 days post-insemination in one study (Davis et al., 1993), and between 26 and 107 days post-insemination in another (Izaike et al., 1991). The entire length of both uterine horns must be carefully scanned to ensure that an embryo is not missed, and care must be taken to ensure that a single embryo was not observed twice.

Table 1. First day of detection of ultrasonically identifiable characteristics of the bovine conceptus (from Curran et al., 1986).

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

Fetal Sex Diagnosis

Transrectal ultrasonography can be used to accurately diagnose the sex of the fetus in cattle based on identification of the genital tubercle (Curran et al., 1989) or the scrotum and mammary gland of the male and female fetus, respectively (Muller et al., 1986). Ultrasonographic location of the genital tubercle allows earlier identification of the sex of the fetus (Days 50 to 100) than detection of the scrotum or mammary glands (Days 73 to 120). The genital tubercle is that which develops into the penis in the male or the clitoris in the female, and is a very prominent structure both grossly and ultrasonographically. The genital tubercle starts out in an undifferentiated position between the hind limbs of the fetus and, beginning on about Day 45, gradually migrates to a position behind the umbilicus in the male or beneath the tail in the female by about Day 55. Under experimental conditions, the experienced operator was able to diagnose the sex of the fetus with 100% accuracy after Day 55 (Curran et al., 1989). Under farm conditions (Curran et al., 1991), an accurate diagnosis was made in 93 of the 97 dairy cows (96%) examined between approximately Days 50 and 100. When the certainty level at the time of diagnosis was high (95 to 99%), a correct diagnosis was made in 65 of 65 animals (100%). Diagnosis could not be made in 5% due to an inability to obtain an adequate view of the genital area in some of the older fetuses (Days 65 to 101). The optimal days for diagnosis were Days 55 to 64, and the time required to make a diagnosis averaged 1 minute 53 seconds (range, 16 seconds to 8 minutes).

■ Imaging the Ovaries – the Bovine Model

Nowhere have the effects of ultrasound imaging been more evident than in our understanding of ovarian physiology (reviewed in Adams et al., 2008). More studies have been published on ovulation and follicular- and luteal-dynamics using the bovine model than in all other species combined (Singh et al., 2003).

One of the best examples of the impact of ultrasonography on our understanding of reproductive function is that of the wave-phenomenon of ovarian follicle development. Controversy surrounded the wave theory of follicular development for nearly 3 decades, until the first ultrasonographic study of the bovine ovaries was reported in 1984 (reviewed in Adams et al., 2008). This new capability created a flurry of interest and led to a series of advancements in our understanding of folliculogenesis and the development of methods to control ovarian function in cattle and other species (reviewed in Adams 1998, 2008). One of the most important advantages arising from these studies is the ability to centralize follicular and hormonal data about a specific ultrasonically detectable point of reference (e.g., ovulation, wave emergence, follicle selection). This capability led to the development of a conceptual model of follicular wave dynamics that provides an explanation of hormonal control of the estrous cycle (Figure 2).

The bovine model, derived from studies in mature nonpregnant cows, has since been extended to other reproductive states and other species. The first study of follicular dynamics during pregnancy and the post-partum period in any species was in cattle. The first study of follicle dynamics during the neonatal and pre-pubertal periods in any species was also in cattle. Since the discovery in cattle, the wave pattern of follicle development has been documented in many other species (camelids, horses, sheep, goats, muskoxen, elk, and humans; reviewed in Adams et al., 2008). Despite the wide range of reproductive patterns represented by these different species (i.e., polyestrous, seasonally polyestrous, single and multiple ovulators, spontaneous and induced ovulators, distinct luteal and follicular phases), the wave pattern of follicle development appears to be a widely conserved phenomenon.

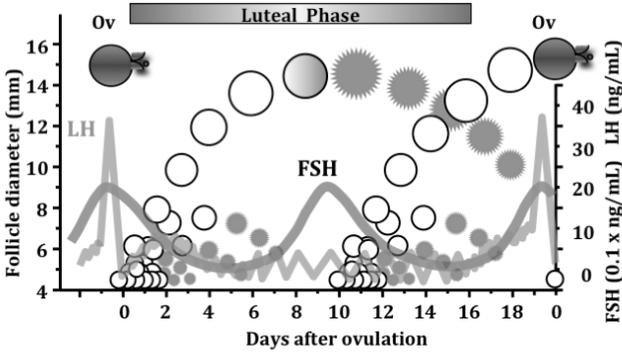
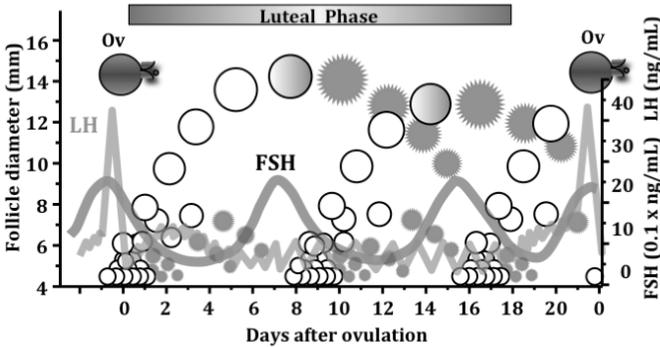
2-wave interovulatory interval**3-wave interovulatory interval**

Figure 2. Ovarian and hormonal dynamics during 2-wave and 3-wave estrous cycles in cattle. Dominant and subordinate follicles are indicated as open (viable) or shaded (atretic) circles.

The majority of bovine estrous cycles (>95%) are composed of either two or three follicular waves. A surge in circulating concentrations of FSH precedes emergence of each wave. A surge in circulating concentrations of LH precedes ovulation. The LH surge is preceded and succeeded by a period of high LH pulse frequency as a result of low circulating concentrations of progesterone (i.e., period of luteolysis and luteogenesis, respectively; Adams et al., 2008). Under the influence of progesterone (e.g., diestrus), dominant follicles of successive waves undergo atresia. The dominant follicle present at the onset of luteolysis becomes the ovulatory follicle, and emergence of the next wave is delayed until the day of the ensuing ovulation. The CL begins to regress earlier in 2-wave cycles (Day 16) than in 3-wave cycles (Day 19) resulting in a correspondingly shorter estrous cycle (19 to 20 d versus 22 to 23 d, respectively). Hence, the so-called 21-d estrous cycle of cattle exists only as an average between 2- and 3-wave cycles. In a recent study (Jaiswal

et al., 2009), the wave pattern was repeatable within individuals, and duration of the estrous cycle was reflective of the wave pattern; i.e., 90% of cycles ≤ 21 days were 2-wave cycles and 80% ≥ 22 days were 3 wave cycles.

■ Computer-Assisted Image Analysis

An ultrasonographic image is composed of thousands of picture elements, or pixels, representing tissue interfaces with a reflectivity ranging from black to white (256 shades of gray in a 8-bit gray-scale image). The human eye can perceive “smoothness” of an image, but can only distinguish between 18 to 20 shades of gray. The status of tissues can be assessed through the use of computer-assisted image analysis.

Spot, Line and Region Analysis

The simplest quantitative method to analyze the pixels comprising an ultrasound image is to select one or multiple small circular or linear areas of interest (Figure 3; reviewed in Singh et al., 2003). The computer can readily determine the precise values of all of the pixels (black = 0, white = 255) within the selected area and provide an average value (mean pixel value) and a standard deviation value (pixel heterogeneity). This simple technique is conceptually similar to the spot metering technique in photography and may be used to compare the pixel values in various portions of the image or among images at different time points. For example, portions of the follicle antrum and wall from sequential images may be evaluated to detect changes characteristic of follicular dominance, growth, atresia, impending ovulation, or luteal development and regression. In line analysis, the amplitude of echoes located along the line may be depicted in a graph (Fig. 4). The antrum-wall interface of the follicle can be detected easily by identifying a sequential rise in grey-scale values along the line. Using the antrum-wall interface as a reference point, pixel values along the line can be divided into three segments, thus allowing separate analyses of the peripheral antrum, follicle wall, and stroma.

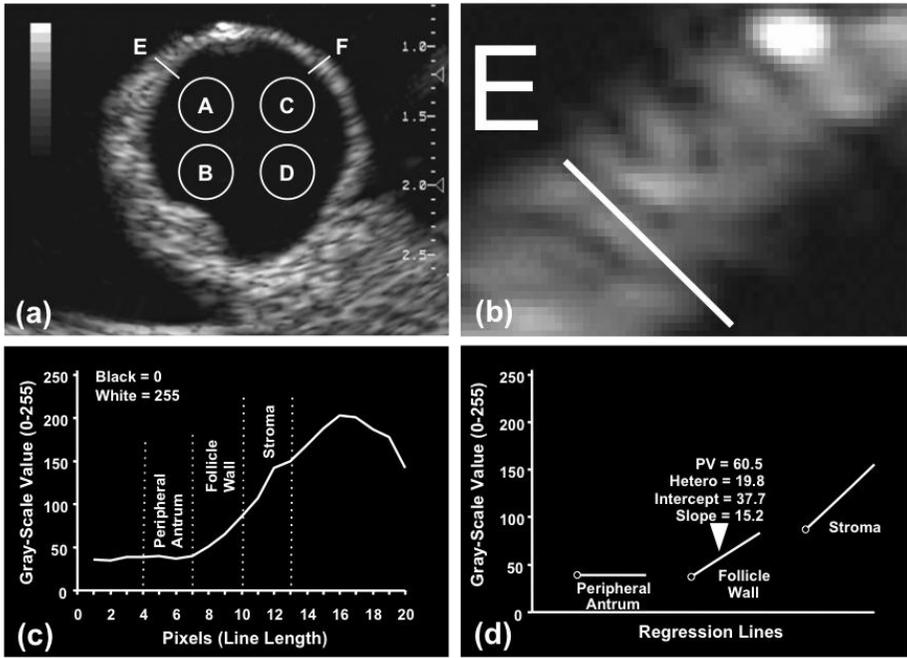


Figure 3. Computer-assisted image analysis of ultrasound images of the ovarian follicle. The follicle wall from (a) is enlarged (b) to display the picture-elements (pixels) forming the ultrasound image. Spot-analysis of the antrum is performed by measuring pixel values within the area of each circle (A,B,C,D). Line-analysis of the follicle wall (b). The pixel value graph (c) was divided into 3 segments (peripheral antrum, follicle wall, stroma) to enable statistical regression analysis of each segment separately. (modified from Singh et al., 1998)

Region analysis of a follicle involves overlaying a pixel-by-pixel “mesh” onto a selected area (Figure 4) for recording pixel values at the points of intersection and generating a 3-dimensional wire-frame model. A computer generated “skin” can be placed over the framework to yield a topographical surface. Shading algorithms (gray-scale or color) may be used to enhance the visual perception of surface features (based on contour and direction) or echo intensities (based on “height” or brightness of pixels) of particular areas. The approach may develop into a diagnostic tool useful in clinical settings permitting instant visual assessment of attributes associated with follicle health.

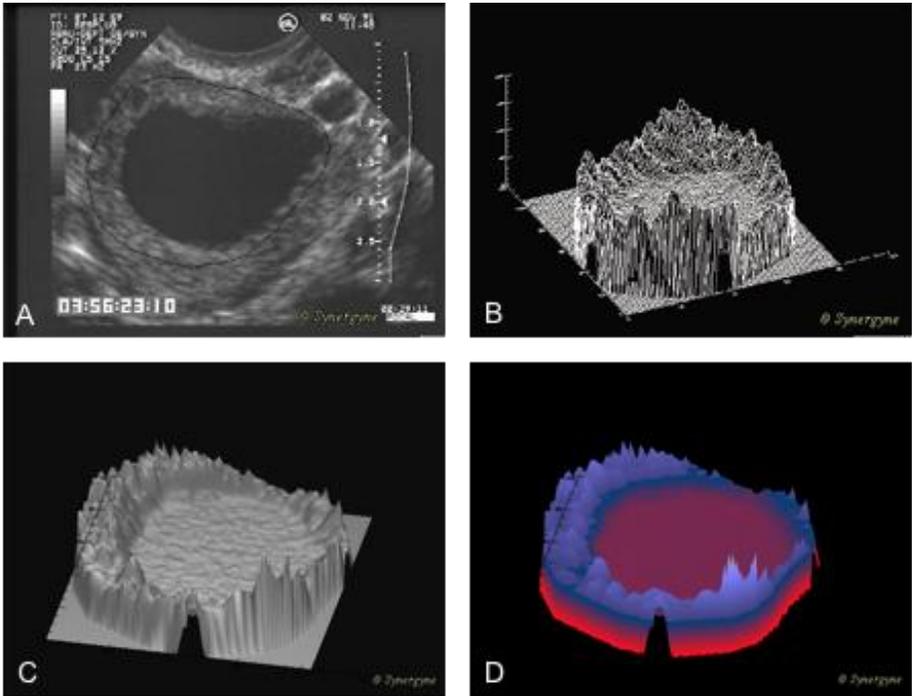


Figure 4. Regional analysis of a single preovulatory follicle. A) Image of the follicle with follicle wall identified (black line). B) Wire-frame model of the pixel by pixel mesh created from the image of the follicle. C) Computer generated “skin” stretched over the wire-frame model. D) Height-shaded color algorithm added to the image to enhance visual appreciation and allow comparison of different zones with images of different follicles or images of the same follicle on different days.

Physiologic Correlates of Image Attributes

In initial studies of high-resolution ultrasound images of bovine ovaries examined at specific phases of follicular wave development (Singh et al., 1998; Singh and Adams, 2000), it was concluded that quantitative changes in the echotexture of ultrasound images occur concurrent with changes in functional and endocrine characteristics of ovarian follicles and CL. The thickness and vascularity of the follicle wall, and the functional status of the CL were reflected in ultrasound image attributes.

Results of studies documenting a relationship between image attributes of ovarian follicles and competence of the contained oocyte to develop into an embryo (Salamone et al., 1999; Vassena 2003) provide rationale for the use

of ultrasound image analysis for identifying follicles that will produce competent oocytes. Although the sensitivity of this technique is not yet sufficient for use in a diagnostic setting, the identification of statistically significant endpoints has formed the basis to improve the imaging technique.

With the objective of developing an image-based classification system that can automatically determine the stage of the estrous cycle based on a single day's ultrasonographic examination of the ovaries, computer algorithms were tested using images of bovine ovaries known to be in either the early (metestrus), middle (diestrus), or late (proestrus) phase of the estrous cycle (Maldonado et al., 2007). Classifier algorithms were 'trained' using a portion of the data set and tested with the remaining portion collected from cows with 2- and 3- wave patterns. The classifiers performed with 86% to 100% accuracy, and although data were taken from only 3 specific periods of the estrous cycle, the results support the hypothesis that the phase of the cycle can be automatically and robustly determined from ultrasound-detected features based on a single day's examination. This constitutes an important step in what could become a fully automated system. The first stage of such a system would be the segmentation of the relevant ovarian structures. If the size of the dominant follicle and size of the CL are a sufficiently rich feature set, then the follicle segmentation problem can be solved fairly easily. Segmentation of follicles ≥ 10 mm has been successful (nearly 100%; reviewed in Maldonado et al., 2007). Segmentation of the CL is the subject of current research (Eramian et al., 2007). For the second step, one need only recognize the largest follicle and measure its diameter. Thus, if future work can achieve a robust segmentation algorithm for the CL, the entire process could be fully automated.

■ Three-Dimensional Ultrasonography

Conventional B-mode ultrasonography involves continuous visualization of two-dimensional "slices" of a tissue (e.g., ovary, uterus, fetus) and mental reconstruction of the tissue structure in the third dimension. This is a technically demanding procedure that has impeded exploitation of the full potential of this type of medical imaging. Advances in computing power and transducer design have now made three-dimensional (3D) imaging possible. A tremendous advantage of 3D imaging is that once the images are acquired and reconstructed, the image may be rotated in all different planes and evaluated without continued scanning. Further, the 3D data sets can be used to provide volumetric measurements and permit acoustic sectioning or stripping of layers of image information, revealing both internal and external features. Apart from computer-related issues, many technical challenges relative to anatomy (e.g., transducer size and configuration for transrectal or transvaginal use) and subject (e.g., animal movement during image acquisition) must be resolved before 3D ultrasonography comes into routine use.

■ Ultrasound Biomicroscopy

The current generation of commercial ultrasound machines is equipped with 3 to 10 MHz probes, and provide a lateral resolution of up to 0.7-1.0 mm. This level of resolution is sufficient for many clinical uses, but the full potential of image analysis can be exploited with an instrument that provides microscopic resolution (i.e., <0.2 mm). An “ultrasound biomicroscope” uses a single crystal to emit a sound wave with a frequency of 25 to 70 MHz and will produce an image with a resolution of 30-50 μm (Jaiswal et al., 2009). Major limitations of the biomicroscope are depth of penetration (approximately 10 mm at 30 MHz, 5 mm at 50 MHz), field of view (imaging width, 1 cm), and frame rate (≤ 8 frames per second). Results of an initial study in cattle (Pfeifer et al., 2010) document the utility of ultrasound biomicroscopy in cattle using a transvaginal approach (Figure 5). The granulosa layer of follicles <1 mm in diameter could be distinguished and the cumulus-oocyte complex was clearly depicted. Echotexture analysis of images obtained with the ultrasound biomicroscope have, for the first time, provided dynamic images of the ovary at histologic resolution.

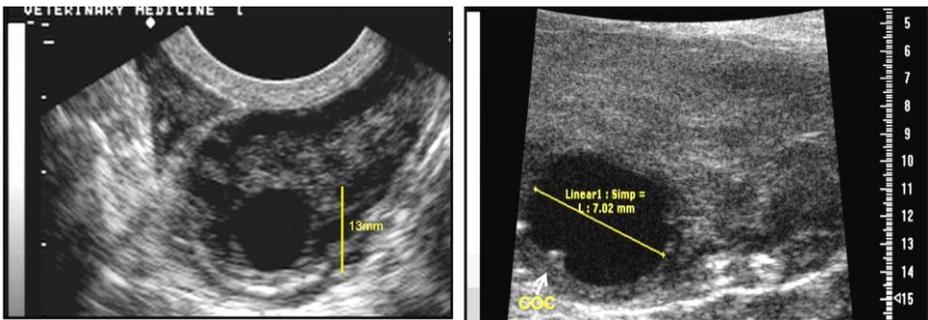


Figure 5. Bovine ovarian follicles imaged by transvaginal ultrasonography using conventional equipment (left; 13 mm follicle; 7.5 MHz probe) and an ultrasound biomicroscope (right; 7 mm follicle; 40 MHz probe). Note the cumulus-oocyte complex in the follicle (COC).

■ Conclusion

Advancements in imaging technologies over the last two decades have ushered a revolution in the study of reproductive structure and function. The most significant advance is in our understanding of the ovary stemming from original studies in cattle. We have developed convincing evidence to support the hypothesis that echotexture characteristics reflect the endocrine and physiologic status of follicles, corpora lutea, and oocytes. Echotexture analysis of images obtained by ultrasound biomicroscopy is the most promising emerging technique for *in vivo* examination of early antral follicles

and oocytes and perhaps, along with 3-D imaging, feature segmentation and classification, we will one day be able to do the equivalent of microscopic and molecular analysis in real-time without removing a tissue sample or interrupting normal function.

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