

## Optical density changes in ultrasonographic images of the endometrium and corpus luteum in pregnant and cyclic cows

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**Abstract:** Optical density image (ODI) analysis, based on the intensity of pixels in digital images, is commonly used in orthopedic medicine. This study was conducted to quantify endometrial and luteal changes in early pregnant and cyclic cows by ODI analysis. Twenty healthy primiparous Simmental cows on days 80–120 postpartum were subjected to ultrasonography and ODI analysis on the days of estrus (day 0) and metestrus (day 4) as well as 12, 15, 18, and 21 days after insemination. The ODI values of pregnant (P) and nonpregnant (NP) cows were compared using two-way ANOVA. The P cows tended to have greater endometrial OD values than NP cows (772.2 vs. 740.5,  $P < 0.08$ ), but their luteal OD values were similar ( $596.0 \pm 24$ ). Both endometrial and luteal OD values varied over time in different patterns in P and NP cows in relation to gestational events until confirmation. In conclusion, preliminary data, especially endometrial OD values on day 15 relative to insemination, could have prognostic merit for a very early detection or confirmation of pregnancy. ODI analysis can be incorporated into ultrasonographic examination. However, more comprehensive experiments are needed to assure the prognostic value of ODI analysis.

**Key words:** Optical density, computer-assisted image analysis, endometrium, corpus luteum, pregnancy, cow

### 1. Introduction

Reproductive ultrasonography in farm animals has advanced since it was first used in 1980 (1). It is an important diagnostic and research tool in domestic (2,3) and nondomestic (4) animals in hospital and field settings. Transrectal ultrasonography was first used for diagnosis of early pregnancy and evaluation of follicle dynamics, hormonal roles of the follicular development, and pathological changes in the uterus and ovaries in mares (5) and cows (6,7).

Technological advancements in imaging and analyzing systems have provided opportunities to elucidate reproductive physiology in veterinary medicine. For instance, some of this information includes the travel of the spherical conceptus in the uterus (8), embryonic elimination in twin sets (9), changes (serration) in the granulosa of preovulatory follicles just before imminent ovulation (10), follicle evacuation during ovulation (11), vascular perfusion of the endometrium during embryonic and placental development (12), fetal sex diagnosis by location of the genital tubercle (13), endometrial echotexture related with the sexual cycle (14), and implantation (15).

Computer-assisted image analysis is one of the most valuable improvements. Recording and analyzing ultrasonic digital images allows quantitative assessments to overcome the subjectivity of visual analysis. This methodology, primarily used in human medicine as a part of evidence-based medicine, was also adapted to farm animal reproduction (14–16). The ultrasonographic appearance or image pattern of an organ is termed “echotexture”. In recent years, computer-assisted ultrasonographic image analysis of B-mode ultrasonography has been used for evaluation of reproductive tissues, including testes (17), ovarian follicles (18), corpora lutea (CL) (19), and uteri (8) in farm animals. Cyclical endometrial echotexture changes in relation to peripheral concentrations of ovarian hormones in cows (14), mares (20), and goats (16). Furthermore, a recent study by Scully et al. (21) showed that pregnant and cyclic cows could be identified according to the uterine echogenicity on day 21 following artificial insemination (AI).

Optical density image (ODI) analysis is another quantitative method, based on the intensity of pixels in digital images (i.e. X-ray and tomography), that is commonly employed in both human and veterinary

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medicine for orthopedic diagnoses such as bone fracture, bone reunion, osteoporosis, and osteomas (22–25). In ODI analysis, each pixel in a standard region named the “region of interest” (ROI) in a digital image is evaluated according to optical density (OD). Incorporation of ODI analysis for ultrasonographic images has not been done in animal reproduction studies. The aim of this study was to assess endometrial and luteal changes in early pregnant and cyclic cows quantitatively using ODI analysis of digitalized ultrasonographic images.

## 2. Material and methods

### 2.1. Cows and experimental setup

The study was carried out on 20 primiparous Simmental cows, which were housed in a commercial dairy farm (32°45'N, 39°53'E) and had no clinical signs of metritis (e.g., asymmetry between uterine horns, cervical enlargement, or abnormal vaginal discharge) on days 80–120 postpartum. The cows were monitored and inseminated artificially upon spontaneous estrus by the same researcher. After insemination on the day of estrus, cows were subjected to pregnancy checks on day 21 relative to the insemination. Cows were then categorized into “nonpregnant” (NP, n = 9) and “pregnant” (P, n = 11) groups. Two cows in Group P were confirmed to be nonpregnant in the ultrasonographic examination for pregnancy on day 35. Their data were excluded in order to avoid interference of possible early embryonic loss. Thus, the study was completed with 18 cows.

### 2.2. Measurements

Real-time ultrasound scanning of the uterus was facilitated via the transrectal route on the days of estrus (day 0) and metestrus (day 4) as well as 12, 15, 18, and 21 days after insemination. Luteal visualization was performed on days 12, 15, 18, and 21 relative to insemination. Transrectal ultrasonography of the uterus was done using a 7.5-MHz linear-array transducer connected to a portable B-mode ultrasound scanner (Agrosan AL, Noveko International Inc., Angoulême, France) as described previously (26). Standardized machine settings [i.e. mode (B), depth (8 cm), MHz (7.5), focus zone (2), B-gain (3), B-brightness (3)] were used throughout the entire study. The same observer performed all ultrasonographic examinations. At least three circular cross-sections of the uterus and CL images (including all layers of the uterine horn) were recorded for each cow on each day of examination (Figure 1).

### 2.3. Optical density image analysis

The ODI analysis was performed on a personal computer using custom-developed software (Quantity One, Bio-Rad, Hercules, CA, USA) as described for digital echotexture analysis of uterine tissues (16). The images were then converted to gray scale and saved in the tagged

image file format (TIFF). Briefly, four predetermined ROIs of 10 × 10 pixels for the endometrium and CL were identified in each of three digitized images for each cow on each ultrasonographic examination. The density of pixels was categorized in the interval between 0 and 2000. The ROI encompassed only endometrial tissue, avoiding myometrial tissue and intrauterine fluid.

### 2.4. Statistical analysis

Prior to statistical analysis, the ODI values for the endometrium and CL were averaged by cow and day. The ODI data were then subjected to one-way ANOVA as repeated measures to compare differences between the NP and P groups and alterations after insemination to confirmation of conceptus (27). Statistical significance was declared at  $P < 0.05$ .

## 3. Results

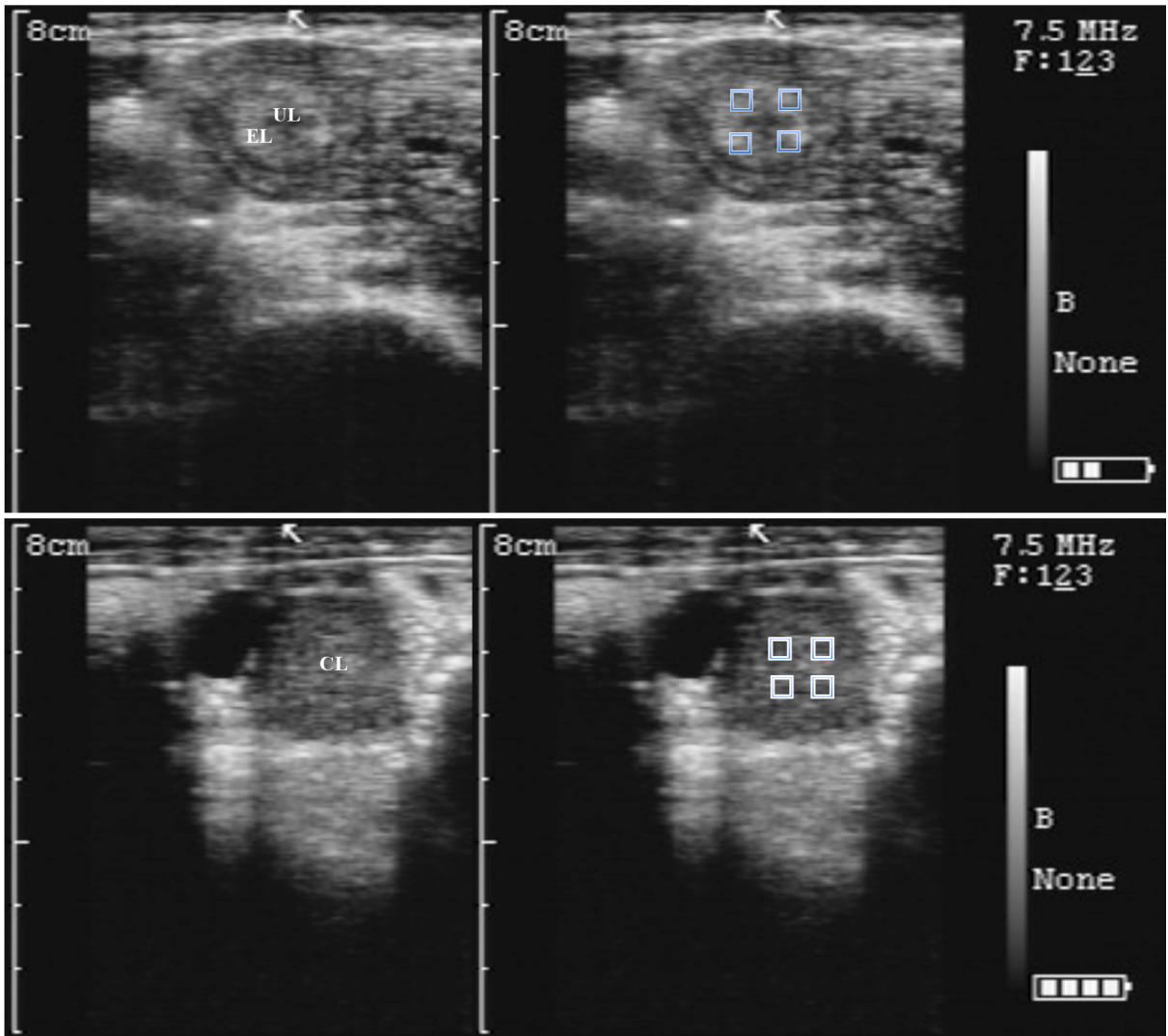
The OD values for the endometrium cubically fluctuated over time within the first 21 days after artificial insemination ( $P < 0.0001$ ; Figure 2A). The mean endometrial OD value for P cows tended to be greater than that for NP cows (772.2 vs. 740.5,  $P < 0.08$ ; Figure 2B). The OD pattern within the first 21 days after artificial insemination for P and NP cows differed ( $P < 0.0001$ ; Figure 2C). The OD value for P cows was higher than that for NP cows in the very early stage (day 4) and the OD value for NP cows was higher than that for P cows later (day 21). On day 15, the OD values decreased in NP cows and increased in P cows.

There was a linear decline from 667.2 to 551.0 in the OD value for the luteal tissue after artificial insemination ( $P < 0.02$ ; Figure 3A). However, the mean luteal tissue OD values for P and NP cows after artificial insemination were similar ( $596.0 \pm 24$ ,  $P < 0.98$ ; Figure 3B). The decrease in luteal OD values for P and NP cows after insemination exhibited a cubical pattern in opposite directions, upward in NP cows and downward in P cows ( $P < 0.03$ ; Figure 3C).

## 4. Discussion

Pregnancy is a complex physiological event and it has economical merit for herd sustainability. Numerous molecules, which are released throughout the estrous cycle and/or during pregnancy, provide a microenvironment for major immune adaptation to accommodate pregnancy. Progesterone, interferon-tau, granulocyte macrophage-colony stimulating factors, prostaglandin- $E_2$ , nitric oxide, pregnancy-specific protein B, and cytokines are the key factors for recognition of conceptus in ruminants (28–30).

In practice, the embryonic vesicle in the pregnant uterus is accurately confirmed by ultrasonography at earliest 21 to 24 days after artificial insemination or natural mating (26). In the case of early embryonic loss, reproductive management strategies are reevaluated and repeated in the following estrous cycle. This eventually



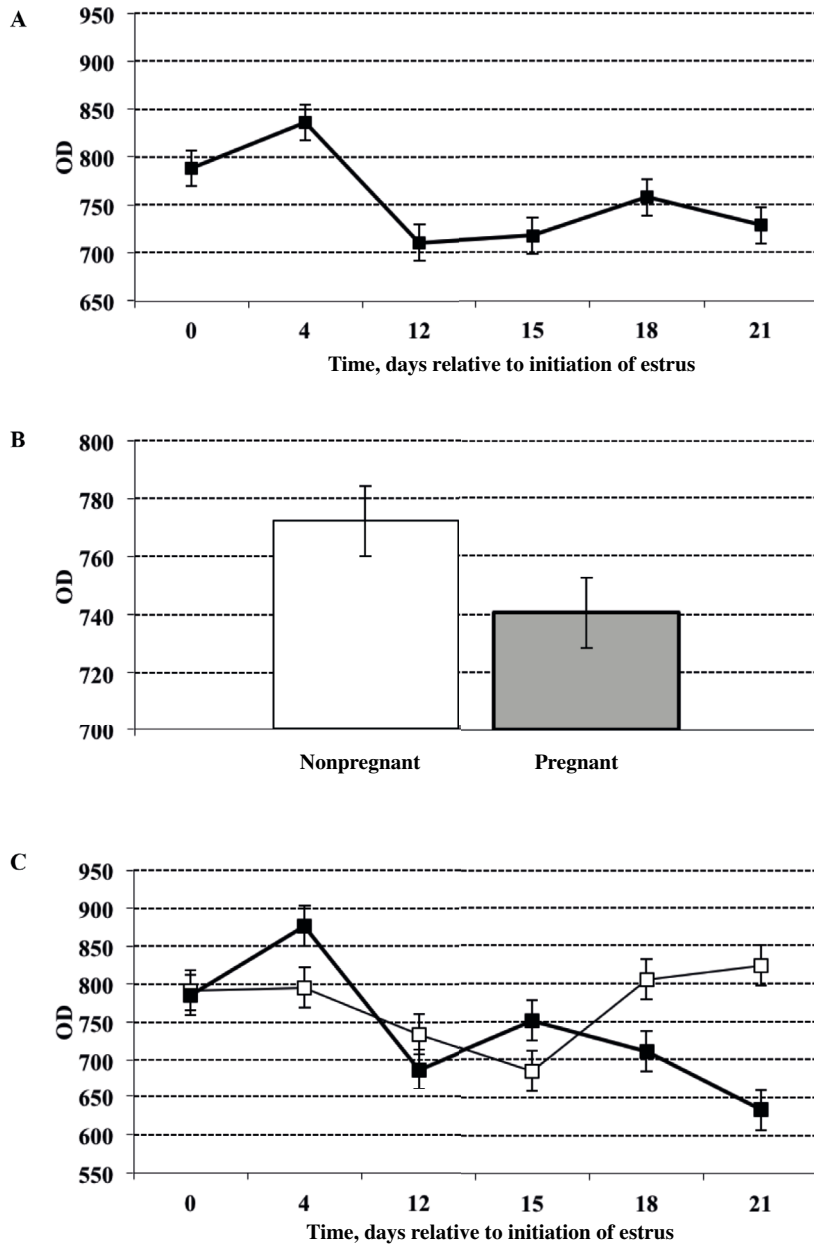
**Figure 1.** Circular cross-section images of the uterus and corpora lutea. UL: Uterine lumen. EL: Endometrial layer. CL: corpora lutea. Squares represent ROIs.

may result in a prolonged calving interval. Thus, much earlier confirmation of pregnancy has great merit in dairy operations. We postulated that OD changes in ultrasonography might predict pregnancy status at a very early stage after artificial insemination/mating.

Profound changes in ultrasonographic appearance, which is related to the estrous cycle, have been reported in previous studies (7, 8). While the echotextural changes in the endometrium were evaluated by visualizing ultrasonographic images in initial studies (7,31) two decades ago, computer-assisted image analysis systems are nowadays used in veterinary reproduction (14,15). By allowing quantitative evaluation, computer-assisted image analysis eliminates individual observation bias. For instance, mean gray level (MGL), homogeneity (HOM), and contrast (CON) are the parameters that are obtained

from ultrasonographic images and define echotextural changes in the uterus (16). The OD value of digital images is a quantitative ultrasonographic parameter as well and is used more frequently in orthopedic medicine. To our knowledge, this is the first experiment adapting ODI analysis to the ultrasonography of bovine uteri to assess early changes in the endometrial and luteal tissues from insemination to confirmed pregnancy.

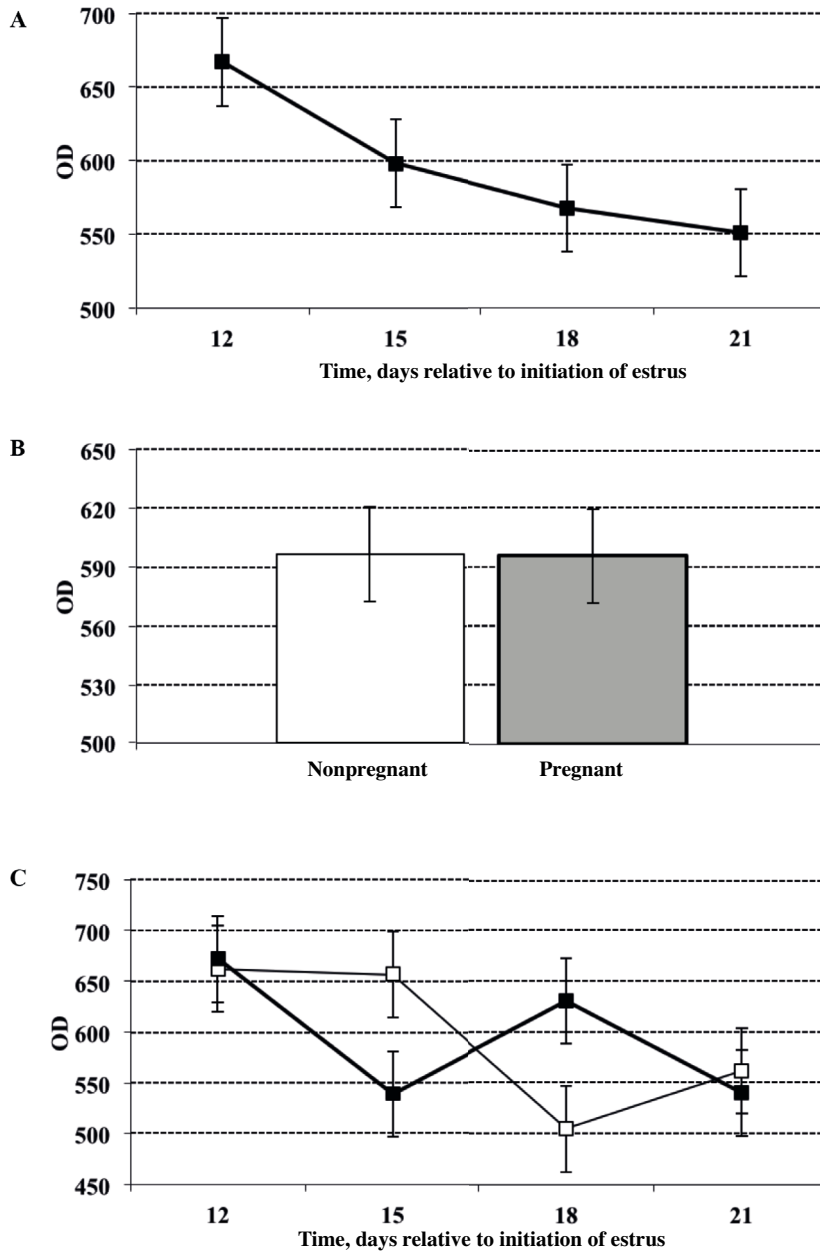
Various hormonal and structural changes occur from insemination/mating to successful conception, which include increased preovulatory activity and early embryonic estrogen concentration, progressive trophoblastic activity, release of secretory vesicles from the endometrium, increased endometrial vascular permeability and edema, and thickened endometrial layers (28,29,31). These biological responses associated with



**Figure 2.** The changes in optical density (OD) in the endometrial tissue of pregnant (-■-, n = 9) and nonpregnant (-□-, n = 9) cows after artificial insemination.

histological alterations in the endometrial tissue can be converted to quantitative measures. Indeed, echotexture parameters change in accordance with reproductive hormonal profiles and embryonic implantation (14). The endometrial echotexture decreases following ovulation, which is linked to edema (7,31). Kauffold et al. (15) reported a notable increase in the MGL value during implantation in pigs. Cengiz et al. (16) also reported a significant increase in the HOM value in pregnant goats 17 days after mating related to the implantation process.

In the present study, the endometrial OD pattern in P and NP cows was different (Figure 2). Early endometrial OD values could be a clue for the initiation of the implantation process. While constant in NP cows, the endometrial OD increased in P cows on day 4 relative to insemination (Figure 2C). Day 4 relative to insemination coincides with initiation of metestrus bleeding in the endometrial layer, which is accompanied by estrogen withdrawal, indicating termination of estrus due to decreased estrogenic activity. This time (about day 4) is the actual start of embryonic development if fertilization of ovum and sperm succeeds.



**Figure 3.** The changes in optical density (OD) in the luteal tissue of pregnant (-■-, n = 9) and nonpregnant (-□-, n = 9) cows after artificial insemination.

The endometrial OD values between P and NP cows were not initially different, but thereafter it continuously decreased in P cows and continuously increased in NP cows (Figure 2C). This may be a sign of embryonic recognition, which is accompanied by changes in a number of biomarkers as mentioned earlier. Moreover, decreased endometrial OD values between days 15 and 21 relative to insemination in P cows could be related to decreased echotexture (32) accompanied by increased glandular secretion, which depends on progesterone

increase (21). However, Schmauder et al. (14) reported no correlation between progesterone level and MGL value. Increased endometrial OD value between days 15 and 21 relative to insemination in NP cows might be associated with increased estrogen:progesterone ratio and uterine tone, indicating imminent estrus (31,32). Likewise, decreased endometrial OD value between days 15 and 21 relative to insemination in P cows might be associated with increased endometrial vascularity (32).

The luteal OD value continuously decreased in a different pattern in P and NP cows after insemination (Figures 3A and 3C). However, the mean luteal OD values for P and NP cows did not differ (Figure 3B). Arashiro et al. (33) also reported a constant level of luteal mean pixel value onset of luteolysis in goats, although it decreased dramatically 48 h later. In a study by Scully et al. (21), it was shown that there were no differences in the luteal echotextures on days 18–21 relative to artificial insemination between P and NP cows. Lacking mean luteal OD differences could be related to the existence of no or a weak correlation between progesterone level and luteal echotextural parameters (14,19,33). Additionally, asynchrony between fast and abrupt decreases in progesterone and slow and gradual decreases in luteal echotextural parameters may explain the lack of differences in luteal OD values between P and NP cows.

In conclusion, the endometrial and luteal OD values were monitored from insemination to pregnancy confirmation. The OD values 15 days after insemination, especially in the endometrial layer, were sensitive to expected biological changes (i.e. metestrus bleeding, implantation process onset), which may have merit for very early detection or confirmation of pregnancy. ODI analysis can be incorporated into ultrasonographic examination. However, this study has certain limitations, such as lacking blood flow, echotexture parameters, and implantation/pregnancy-related biomarkers. Therefore, comprehensive experiments are needed to claim the merit of endometrial OD values for very early confirmation of pregnancy.

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