Bovine follicular and luteal blood flow during the estrous cycle

Clinical evalutation of the ultrasound Doppler technology

Mariska Lansbergen
Student number: 3515710

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Contents

Abstract

Color Doppler technique in cattle reproduction is a useful tool to visualize physiological and pathological processes of the reproductive system. The objective of this study is to make an evaluation of the blood flow during follicular and corpus luteum development during the estrus cycle and find a correlation between the blood flow, progesterone levels and size during the cycle. Six Holstein-Friesian cows are synchronized and examined sonographic during the estrus cycle and progesterone levels are measured every other day. Ovulation is induced using prostaglandins at day 14. The maximal cross-sectional size of the follicles and corpora lutea are measured sonographic, the blood flow to follicles and corpora lutea are examined using Color Doppler images. A correlation of r=.351 between follicle growth and blood flow for follicles larger than 10 mm is found. The blood flow and progesterone levels are correlated during growth, static and regression phase, where progesterone and luteal size are only correlated during growth and regression phase. Over the complete cycle a correlation of r=.788 is found between blood flow and progesterone levels (r=.766 for progesterone and luteal size). This indicates that blood flow can give a reliable estimation of the functionality of the corpus luteum. It is concluded that the ovarian blood flow is a reliable research method, which gives useful additional information in fertility counseling.

Introduction

Doppler echo is a relatively new technique in the reproduction of cattle. However it is already often used in cardiology and has proven its clinical usage. In human reproduction the technique is used to examine the prognosis of an embryo implantation and survival after an embryo transfer in IVF programs (Steer et al, 1994) or in high risk pregnancies (Dickey, 1997). The blood flow measured in the ovaries and uterus can give important information on the development of the fetus (Steer et al, 1994).

In cattle the application of Doppler sonographic echo can be very useful, a pregnancy can be diagnosed significantly earlier compared to rectal palpation, for example (Matsui and Miyamoto, 2009). Uterine blood flow increases after ovulation in pregnant cows, non-pregnant cows show a decrease in blood flow after ovulation (Ford et al, 1979). From 28 days of pregnancy, extra cardiac fetal blood flow can be seen, and blood flow in the umbilical cord, aorta and carotid artery can be observed from 38 days (Miyamoto et al, 2006). Moreover, Doppler echo can be used to visualize fetal loss, a situation where the blood flow to the corpus luteum decreases rapidly before the next estrus (Matsui and Miyamoto, 2009).

During the estrus cycle Doppler echo can also be used to examine the ovaries, for example to monitor the blood flow in growing follicles or the corpus luteum or to support the diagnosis to discriminate between a follicular or luteinized cyst. The luteinized cyst produces substantial levels of

progesterone, whereas the follicular cyst produces lower or basal amounts of progesterone (Douthwaite and Dobson, 2000). Ultrasonography is used to determine the thickness of the cystic (luteinized) wall and to learn whether it is a follicular or luteinized cyst; the Doppler echo technology provides supplementary information about the degree of blood flow making the diagnosis more easily and therefore more accurate (Matsui and Miyamoto, 2009).

The possibilities of the practical use of Doppler echo are still in development and are promising in bovine reproduction. The equipment has decreased in size over the last few years and is therefore more practical for the usage on farms. Since the prices of the machines have decreased as well, the practical use of this technology for the practitioner improves during the next few years (Herzog and Bollwein, 2007).

To work accurately with the Doppler equipment, expertise and experience are required, but also knowledge of the changes during the estrus cycle and during pregnancy. In the clinic of Farm Animal Health and the field the use of Doppler is still limited and therefore an evaluation of the usage of Doppler echo is required and may be valuable.

The experiment is preformed by a researcher with limited experience in sonographic imaging and Color Doppler technology. The results therefor can be used by practitioners, inexperienced with Doppler technology, or educational settings, for the skills are comparable with those of the researcher. The evaluation therefor is very useful for practitioners who consider using this new technology.

During this research an evaluation of the blood flow during follicular and corpus luteum development during the estrus cycle will be made and the correlation between follicular size, progesterone levels and blood flow in the corpus luteum and dominant follicle will be calculated. This correlation may be indicative for practitioners using the Doppler technology in the field.

Uterine blood flow

Estrus

Sonographic imaging of the uterus is widely used in ruminant reproduction and since roughly 13 years the Color Doppler technique has been introduced (Bollwein et al, 2000). The uterine artery can be located using the rudimentary umbilical artery and the blood flow through it can be measured. When using high frequency transducer high Doppler shifts can be found, which can be reproduced quite precisely (Herzog and Bollwein, 2007). The blood flow to the uterus during the estrus cycle has a specific pattern; it is relatively stable during the diestrus, and rises during pro-estrus and estrus (Bollwein et al, 2000).

It is thought to be influenced by estrogen levels in the blood, since estrogen is considered to be a vasodilatator (Mattioli et al, 2001). According to

Bollwein (2000), estrogen does influence the blood flow, but the correlation with the blood flow was only r=.51 (Bollwein et al, 2000), which indicates that other factors have an influence as well (Herzog and Bollwein, 2007). Bollwein also found a negative correlation between progesterone and the blood flow (r=-.32) (Bollwein et al, 2000), only 10 percent of the changes in blood flow to the uterus during the cycle can be explained by the changing progesterone levels (Herzog and Bollwein, 2007).

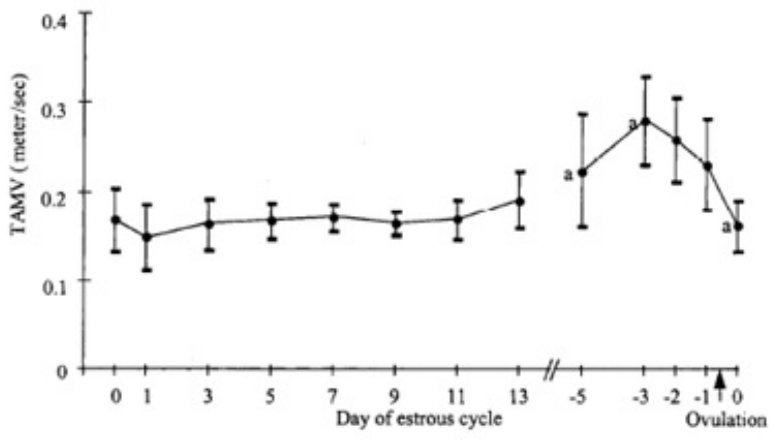

Figure 1: Time-averaged maximum velocity (TAMV) from the Aa. uterinae. Values are the means ± SD of 4 cows and 2 estrus cycles. The values with the letter 'a' differ from those of the previous measurements ($p<0.05$) (Bollwein et al, 2000).

Gestation

The blood flow to the uterus during pregnancy changes as well. The growth of a fetus is exponential (Ruesse and Grunert, 1993) and during growth the fetus needs oxygen and nutrients. Therefore, more blood is required to supply the uterus. The blood flow to the uterus in the ipsilateral horn rises exponential along with the growth of the fetus (Bollwein et al, 2012). In the contralateral horn the blood flow also increases during pregnancy, but further to the end of gravidity the difference between blood supply to the horns grows larger (figure 2) (Bollwein et al, 2012).

Puerperium

The uterus involutes rapidly after parturition, in four days the size of the uterus decreases significantly (Gier and Marion, 1968). During this involution the blood flow decreases; within approximately 28 days after parturition the blood flow is back at the base level (Krueger et al, 2009). Cows with problems during the first week post partum show a less rapid decrease in blood flow to the uterus. The drop in blood flow occurs approximately four

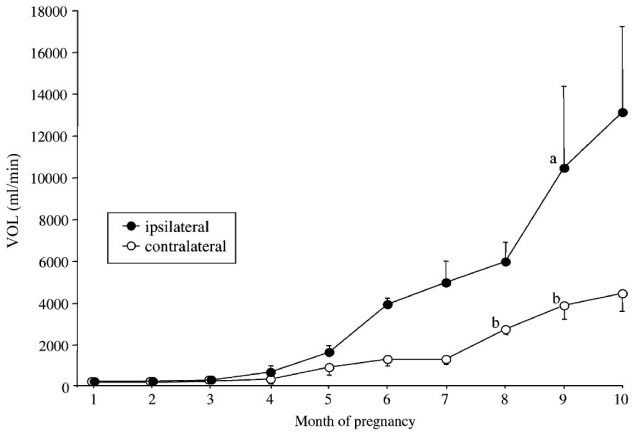

Figure 2: Volume of blood (VOL) in uterine arteries ipsi and contralateral to the conseptus. Values are means ± SD of 3 cows. The values with the letter (a, b) differ from those of the previous measurements ($p<0.05$) (Bollwein et al, 2002)

days later than in a normal puerperium (figure 3) (Leidl, 2000). This delay may be caused by a higher level of prostaglandin due to endometritis since Slama et al (1994) found high levels of prostaglandins in cows with endometritis (Slama et al, 1994) and they are considered to be a vasodilator in sheep (Redsnik and Brink, 1978). Another possible explanation may be the hyperemia due to inflammation, as was found in women with abnormal puerperal courses (Kirkinen et al, 1988).

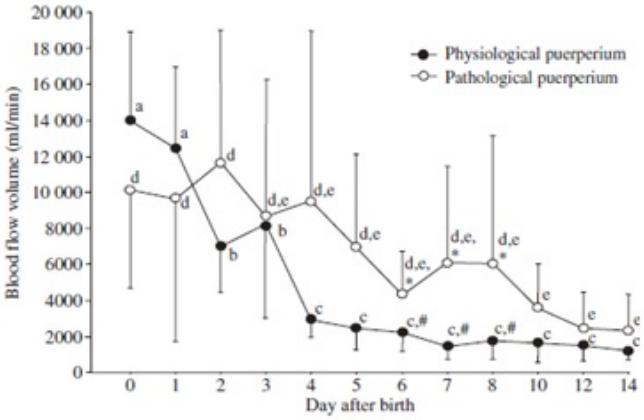

Figure 3: Uterine blood flow volume during the puerperium in cows. Values are mean ± SD of four cows each with a physiological and a pathological puerperium. Values of the same parameters with different letters (ae) differ between days ($p<0.05$). Values of different parameters with different asterisks (∗, #) on the same day differ ($p<0.05$) Day 0 is 3 h postpartum (Leidl, 2000).

Ovarian blood flow

Estrus, follicular phase and ovulation

During growth, follicles are supported by a blood network. The blood flow of the follicles, detectable with Doppler technology, is similar for the middle and large follicles before deviation (Acosta et al, 2005). The production of estrogen and inhibin by the large follicles inhibits the growth of the other follicles. The larger follicles still grow despite the lower Follicle Stimulating Hormone (FSH) levels (Senger, 2003). After selection the blood flow to the dominant follicles increases significantly more than the blood flow to the middle or small follicles. The blood flow to the large follicles is visible at the point of deviation; there is a significant difference in detectable blood flow between the dominant follicle and the second largest follicle after deviation (figure 4) (Acosta et al, 2005). This suggests that the blood flow around a follicle is important for the growth of a dominant follicle (Matsui and Miyamoto, 2009).

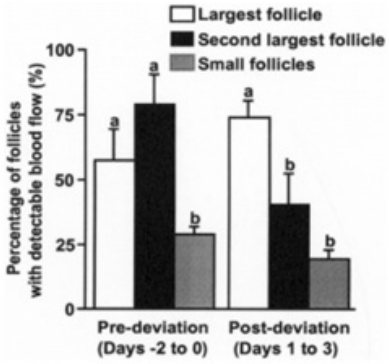

Figure 4: Change in the percentage of follicles with detectable or undetectable blood flow around the day of follicle deviation. Different letters indicate differences ($p<0.05$) between groups (Acosta et al, 2005).

Ovulation is induced by a combination of a Luteinizing Hormone (LH) surge and local factors which reach the dominant follicle through a fine network of capillaries (Acosta and Miyamoto, 2004). After the ovulation a corpus hemorrhagicum remains (Senger, 2003). The increase in blood flow to the remains of the follicle is probably due to LH, as found in rats (Varga et al, 1985), rabbits (Janson, 1975), and sheep (Niswender et al, 1976). Acosta and Miyamoto (2004) also found an increase in blood flow at the same time as the LH surge in cows, which indicates that LH plays an important role in the conservation of the blood flow (Acosta and Miyamoto, 2004).

Luteal phase-corpus luteum

During the days following the ovulation, the luteal size increases, as does the production of progesterone, which are correlated during the growth period (r=0.69) (Herzog et al, 2010). The growth occurs very rapid and is induced by insulin like growth factor (IGF). The development of the corpus luteum (CL) and its functions are dependent on blood supply (Acosta and Miyamoto, 2004). This blood flow is possible due to angiogenic growth factors as Fibroblast Growth Factor and Vascular Endothelial Growth Factor. This makes the high rate of cell turnover and high metabolic rate possible (Revnolds and Redmer, 1999). After the development of the corpus luteum, there is a static fase in the luteal size. In this period the progesterone production still increases, as does the blood flow to the corpus luteum (Bollwein et al, 2012).

The regression of the corpus luteum is regulated by the production of PGF2 (Senger, 2003). After the release of the prostaglandins (or when injected intra muscular) the blood flow to the corpus luteum increases rapidly and almost doubles, before decreasing significantly within two days (Miyamoto et al, 2005). This increase in blood flow is caused by Nitric Oxide (NO) which is produced by endothelial and fully luteinized granulosa cells, under the influence of PGF2. NO induces vasodilation of the arterioles and increases the blood flow to the corpus luteum. This increase leads to shear stress in endothelial cells of microcapillary vessels. These cells and PGF2 induce endothelin I (ET I) and angiotensin II (Ang II) secretion in the corpus luteum. These are both vasoconstrictive peptides and cause long-term vasoconstriction. PGF2 may also be responsible for down regulation of Nitric Oxide Synthase (NOS) during luteolysis (Miyamoto et al, 2005). Due to this mechanism (see figure 5) the blood flow decreases rapidly during the regression phase.

The progesterone levels also decrease rapidly during regression phase, and is correlated to the blood flow (r=.77) (Herzog et al, 2010). The decrease in progesterone may be caused cytokines which are originated from fibroblasts, endothelial and epithelial cells of the corpus luteum. They inhibit the LH-stimulated progesterone production of the luteal cells. IGF1 is a luteotropic factor and stimulates the progesterone secretion. TNF and IL1b elevate the IGF-binding protein and therefore the availability of IGF is less. IGF also inhibit the Fas-mediated cell death (Neuvians et al, 2004). The Fas-ligand binds to the Fas (CD29) and induces clustering, death domains bind and death effector domains on the Fas and recruit pro-caspase 8. The pro-caspase cleaves and activates itself, to be released and activate a downstream effector caspases and induce cell death (Murphy et al, 2008). This leads to more apoptotic luteal cells due to more cytokines (Neuvians et al, 2004). All these processes combined cause the luteal size, the progesterone production and the blood flow to decrease within a few days.

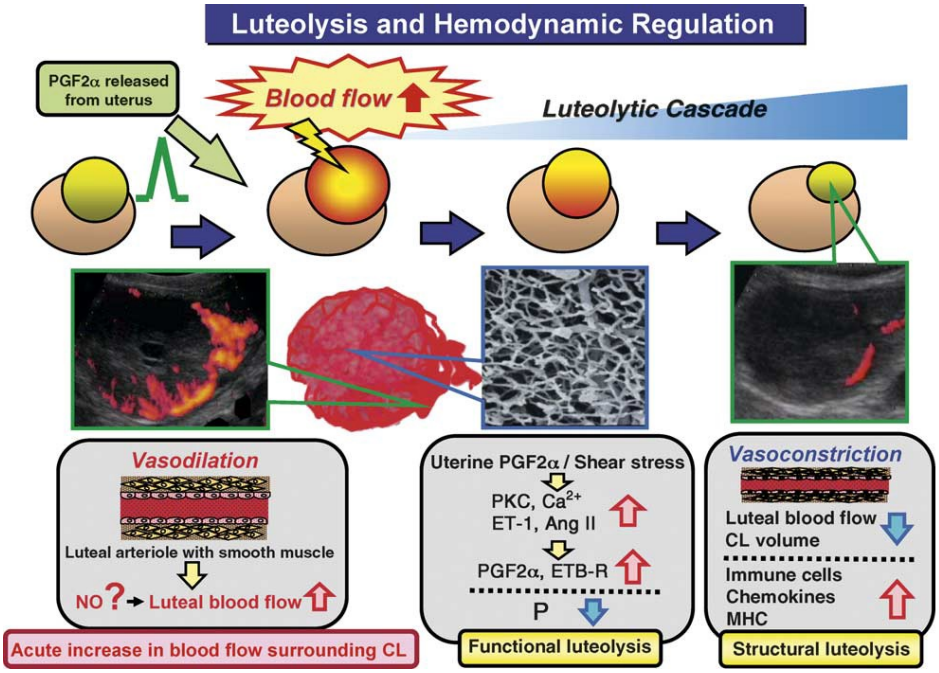

Figure 5: Proposed concept of luteolysis triggered by an acute increase in the local blood flow (BF) of the CL. PGF2 released from uterus stimulates nitric oxide (NO) production and release in the arterioles of the peripheral vasculature of the mature CL, inducing vasodilation of the arterioles. These site-restricted mechanisms increase the BF to the CL. This acute increase in BF triggers the cascade of luteolysis. The increase in luteal BF may be induced by NO from large arterioles surrounding the CL to induce high shear stress, and simultaneously uterine or exogenous PGF2 directly increases ET-1 and Ang II secretion from microcapillary vessels within the CL. These vasoactive peptides secretion from neighboring luteal cells directly and may later induce chronic vasoconstriction of the arterioles of the CL. Subsequently, intra-luteal PGF2 increases, thereby causing a gradual decrease in P production. Structural regression of the CL is indicated by a gradual reduction in CL volume and luteal BF, accompanied by immune cell infiltration, increased in chemokines, and expression of major histocompatibility complex molecules (MHC) within the CL to accelerate and ensure tissue destruction (Acosta et al, 2005)

Gestation

A fertilized oocyte develops into a blastocyst and starts producing interferon-intrauterine. This interferon- inhibits the formation of oxytocin receptors on the uterine tissue. Without these receptors, no PGF2 will be produced and luteolysis will not be initiated. The corpus luteum stays intact and continues to produce progesterone to support the pregnancy (Senger, 2003). The small increase of blood flow to the corpus luteum found in pregnant cows (Herzog et al, 2011) enables the continuance of the progesterone production.The non-pregnant or non-bred cows show a decrease in the blood flow to the corpus luteum after fifteen days, compared with pregnant cows (figure 6) (Herzog et al, 2011).

The blood flow to the corpus luteum in pregnant cows, during the first three weeks, is significantly higher than in non-breed or non-pregnant cows. However the differences between pregnant cows and cows with early embryonic loss during the first three weeks, is not significant (Herzog et al, 2011). This may be due to large differences in blood flow between cows (Herzog et al, 2011). The diagnosis of early embryonic loss in the first three weeks therefore cannot be made using Doppler echo (Bollwein et al, 2012). However the embryonic loss later in the pregnancy can be detected by Doppler echo, since the blood flow to the CL in a pregnant cow is high and decreases rapidly after loss of the fetus (Matsui and Miyamoto, 2009).

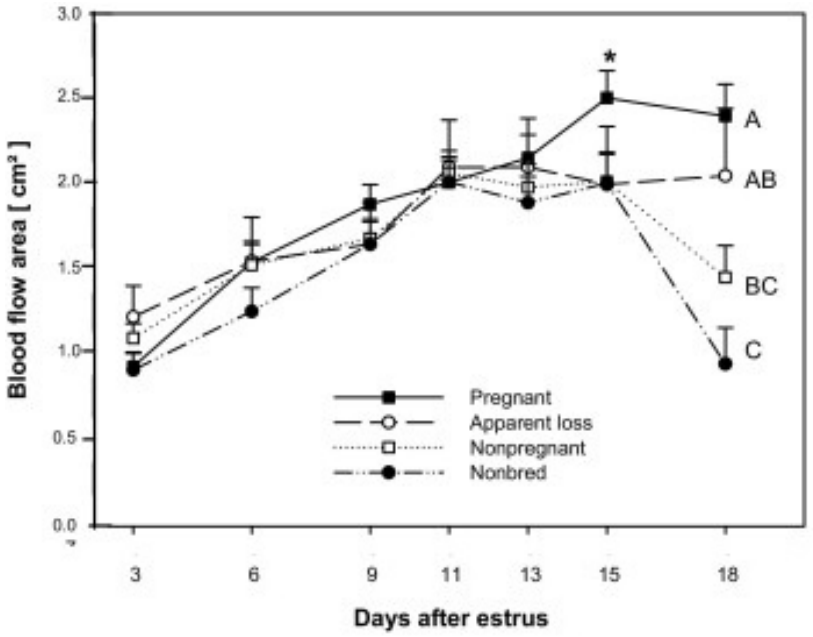

Figure 6: Mean luteal blood flow in dairy cows on Days 3 to 18 (estrus = Day 0). Luteal blood flow. There were effects of group, time, and a group by time interaction ($P<0.05$, $P<0.001$, and $p<0.001$, respectively) (Herzog et al, 2011).

Ovarian Cysts

Follicles of 25 mm or more, which persist for more than ten days in absence of a functional corpus luteum, are diagnosed as cysts. Cysts are found on ovaries quite often and are an important aspect of fertility evaluation. When cysts are luteinized, the walls of the follicle are thicker (more than 3mm) and the follicle produces significant levels of progesterone (Douthwite and Dobson, 2000). The treatment of a luteinized cyst is an injection of PGF2, which will lead to estrus within 8 days (Peter, 2004). When cysts do not have luteinized walls, they are called follicular cysts. The treatment of a follicular

cyst is injection of GnRH, which will lead to ovulation. The usage of GnRH to treat a follicular cyst leads to ovulation in 18 till 25 days, for the cycle will be initiated (Kesler and Garverick, 1982). Therefore the differentiation between a luteinized and a follicular cyst is very important, to determine the most optimal treatment. Evaluation of the cysts by ultrasonographic imaging can be used to diagnose between the types of cysts. The Doppler technology shows the blood flow in the wall, and the area of the blood flow therefore gives a clearer view of the thickness of the wall (figure 7). This makes the choice for therapy and advice easier (Matsui and Miyamoto, 2009).

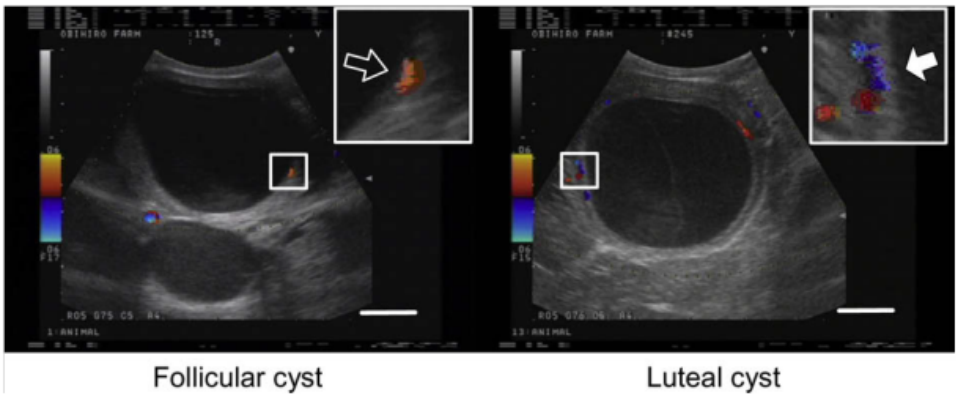

Figure 7: Blood flow in ovarial cysts (Matsui and Miyamoto, 2009)

Materials and methods

The study is completed at the University Utrecht, the department of Farm Animal Health in the Netherlands between September 2013 and November 2013. Eigth cyclic Holstein-Friesian cows from the experimental herd are used, kept under the normal management of the Veterinary Faculty. The cows are fed with silage and concentrate, with free access to water.

Synchronization of the estrus cycle is achieved using Controlled Internal Drug Releasing devices (CIDR Device protocol, the Netherlands, Zoetis) at day -9 (estrus day 0). At day -3 an injection of PGF2-analog is admitted and at day -2 the device is removed from the uteri. One to three days after removal of the CIDR device the cows are in estrus, where the second day is determined as day 0. After synchronization two cows are excluded based on their reaction on the synchronization-protocol, and six cows continue in the experiment. The estrus cycle is subdivided into three phases: luteal growth phase (day 4 to 7), luteal static phase (days 8 to 14) and luteal regression (day 14 to 19). To synchronize the luteolysis as well, a prostaglandin (5 ml Enzaprost, im) is injected at day 14 (figure 8)

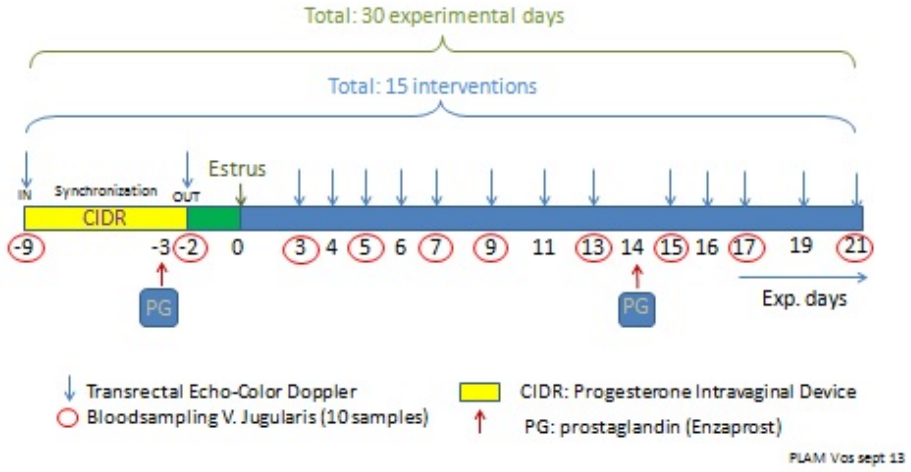

Figure 8: Experimental design with transrectal Color echo, blood sampling from vena jugularis, Controlled Internal Drug Releasing device and prostaglandin injections indicated

Sonographic investigations are done by one operator between 8:00 and 12:00 a.m. with a color Doppler ultrasound device (Mylab Vet, ESAOTE, Maastricht, The Netherlands). These investigations are done daily during luteal growth and luteal regression and every second day during luteal static phase, so examinations take place on day 3, 4, 5, 6, 7, 9, 11, 13, 15, 16, 17, 19 and 21. Also during the CIDR two echo-graphic examinations are conducted at day -9 and -3.

Images are taken from the maximal diameters of the respective corpus luteum or follicles of 10 mm or larger. The blood flow will be semi-quantitatively determined using the quantity of colored pixels within the area of interest (corpus luteum or follicle). This is done using three images (saved on USB) of the largest follicle and corpus luteum with color Doppler and three images with Power Doppler. To exclude variations in recording all Doppler system controls are pre-installed (Frequency: 5 MHz, PRF Color: 750 Hz, Smooth: 0, Power : Max, Wall filter: medium, Persistence: 3, Sensitivity: medium, Max Threshold: 3, Colormap: 3, Baseline: 50 %).

The diameters of corpus luteum and follicles and the colored pixels are evaluated using an off-line program (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). For all measurements is the mean of the three images is used for further calculations. Since not all cows were synchronized correctly, three of the cows where retrospectively synchronized based on plasma progesterone levels over time. The measurements of day 5 are used as a basal value (100 percent). The retrospective synchronization inhibited to use day 3 and 4, because there were no results obtained from every cow.

Blood samples are taken at the start of the experiment, at the removal of the CIDRs and every other day from day 3 till day 21 (day -9, -2, 3, 5, 7, 9, 11, 13, 15, 17, 21). The blood is withdrawn from the jugular vein and the progesterone concentrations are determined by radioimmunoassay analysis in the plasma.

All means of the measurements per cow are used to calculate a mean percentage of progesterone concentration, follicle size, luteal size, luteal blood flow and follicular blood flow from all investigated cows, using an excel-spreadsheet (Office Excel 2013, Microsoft Corporation). Relative changes are also calculated, with the results from day 5 as 100 percent.

The information obtained is used for statistical analyses using SPSS (version 20; SPSS Inc., Chicago, IL, USA). Statistical correlations between follicular size and follicular blood flow, between luteal size and luteal blood flow, between progesterone concentration and luteal blood flow and between progesterone concentrations and luteal size were quantified by Pearson correlation coefficients (all with $P<0.01$). Significant differences in relative changes between progesterone levels and luteal size or luteal blood flow, respectively are calculated using paired sample t-test.

Results

Animals

Eight cows started the experiment and received treatment for synchronization of the estrus cycle. Of these selected animals only three cows ovulated after the treatment, based on plasma progesterone levels and sonographic imaging. Apart from these three cows, three animals were selected for the experiment, based on cyclicity and ease during examination. The results obtained from these animals were retrospectively synchronized with the other cows.

Follicles

All follicles of 10 mm diameter or bigger were examined, one of the follicles bigger than 10 mm diameter in each cow was monitored. These follicles showed a growth from up to a mean of 19 mm in approximately 6 days. On average the blood flow of the follicles of 11 mm diameter was 66.6 colored pixels and increased up to 127 colored pixels. However the highest found blood flow was 266 colored pixels corresponding with a diameter of 15 mm, the largest found diameter was 20 mm with a corresponding blood flow of 187 colored pixels. As shown in figure 9 the results were disseminated and only a part of the results were explained by the trend-line drawn in the figure 9. The correlation between the follicular size and amount of colored

pixels was r=.351, indicating that the relation between size and blood flow was not very strong.

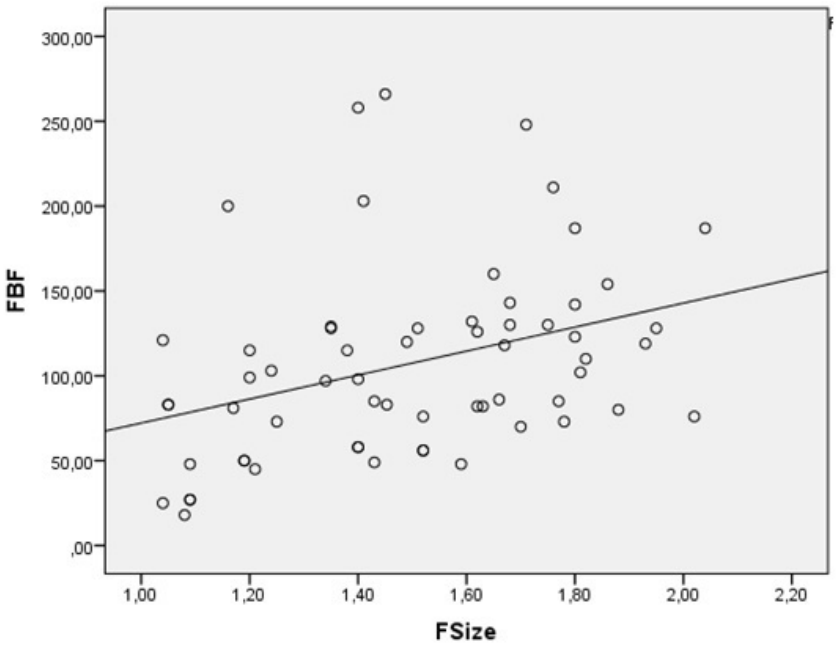

Figure 9: Measurements of amount of colored pixels plotted against corresponding the follicle size, values of six cows, with a regression line of r=.351

Corpus Luteum

The average luteal size started at 20 mm at day 5, the diameter increased till 28 mm on day 7 (the luteal growth phase). During the static phase the corpus showed less growth, from 28 mm on day 7 till 31 mm on day 13. The prostaglandin injection on day 14 initiated the luteal regression phase, where the corpus luteum decreased in size to 17 mm on day 16. At day 21 the growth started again.

The plasma progesterone levels rose during the growth phase from 0,3 ng/mL average on day 3 till 2,6 ng/mL on day 7 and continued rising in the static phase. A mean maximum of 5,4 ng/mL was found on day 13. Starting on day 15 the plasma progesterone decreased quickly to a mean level of 0,1 ng/mL on day 17. The levels began to rise again on day 21 (0.6 ng/mL).

The luteal blood flow showed a large increase in the growth phase (1738 colored pixels on day 5 and 3086 colored pixels on day 7). The flow kept increasing in the static phase till day 13 on which the blood flow was 6540 colored pixels. This is an increase of 212 percent. After the prostaglandin injection the blood flow decreased to 732 colored pixels on day 16. The

blood flow rose again on day 21 (157 colored pixels).

Day	Mean CI Size (SD) cm	Mean CL BF (SD) pixels	Mean P4 (SD) ng/mL
5	1.96 (± 0.42)	1738 (±839)	0.97 (± 0.41)
7	2.78 (± 0.32)	3086 (±602)	2.63 (±0.75)
9	3.03 (±0.43)	3768 (±1301)	3.92 (±0.92)
11	3.09 (±0.45)	5077 (±2160)	4.25 (±1.10)
13	3.11 (±0.38)	6540 (±2122)	5.43 (±1.36)
15	2.3 (± 0.34)	2388 (±1230)	1.05 (±0.96)
17	0.31 (± 0.58)	81 (±242)	0.12 (±0.04)
19	0	0	0,03 (±0.05)
21	0.41 (±0.63)	157 (± 260)	0.57 (±0.57)

Figure 10: Means ±SD diameter of the corpus luteum (in cm), means ±SD of colored pixels in the corpus luteum, means ±SD of plasma progesterone levels (in ng/mL), values are of six cows.

Day	Mean CLSize (SD) %	Mean CL BF (SD) %	Mean P4 (SD) %
5	100	100	100
7	134 (±15)	167(±44)	302 (±95)
9	157 (±26)	234 (±68)	458 (±153)
11	160 (±24)	308 (±88)	508 (±223)
13	161 (±20)	400 (± 88)	680 (±359)
15	119 (±18)	142 (±52)	116 (±84)
17	11 (±27)	3 (±6)	15 (±10)
19	0	0	3 (±3)
21	22 (±34)	14 (±21)	54 (±53)

Figure 11: Means ±SD of relative changes in diameter of the corpus luteum (in percentages), means ±SD of relative changes in colored pixels in the corpus luteum (in percentage), means ±SD of changes in plasma progesterone levels (in percentages), values are of six cows.

Relative changes were calculated to exclude the differences between cows, the results of day 5 were defined as 100 percent. When these relative changes were visualized (figure 13) the changes in the luteal growth phase show a similar pattern as the changes in the progesterone levels. The correlation between the blood flow and progesterone levels, the blood flow and luteal size and the progesterone levels and luteal size were r=.710, r=.928 and r=.747,

respectively. When relative changes were used to determine the correlation, the correlations are r=.818, r=.816 and r=.735.

However, the static phase showed differences between the increase of the luteum size (13 percent increase) and the blood flow and plasma progesterone levels with an increase of 69 percent and 94 percent respectively (figure 12). During the static phase the correlation between blood flow and progesterone was r=.201, when calculated from the relative changes r=.378. A correlation between the blood flow and luteal size was only r=.344 (r=.159 for relative changes). Between the progesterone levels and luteal size no correlation was found.

At day 15 the regression phase started and the blood flow and progesterone levels dropped quickly within two days (about 90 percent). The correlation found between the blood flow and progesterone levels was r=.441 and r=.710 for the relative changes. Between the blood flow and luteal size a correlations of r=.875 and r=.862 respectively were found. The progesterone levels and luteal size showed a correlation of r=.555 or r=.713 when calculated with relative changes.

Over the complete estrus cycle the correlation between the blood flow and progesterone levels is r=.788. Between the blood flow and luteal size was a correlation r=.818 measured over the complete cycle. And the found correlation between the progesterone levels and luteal size is r=.766. When the relative changes are used, the complete cycle correlates r=.818, r=.816 and r=.735 respectively.

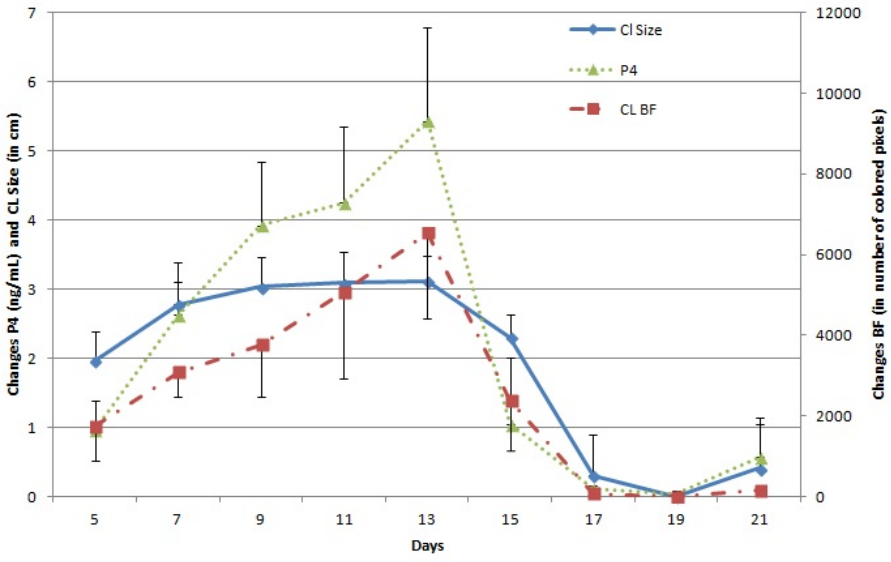

Figure 12: Mean ±SD of blood flow (in number of colored pixels), ±SD of progesterone levels (in ng/mL) and ±SD of the diameter of corpus luteum (in cm) during the experiment

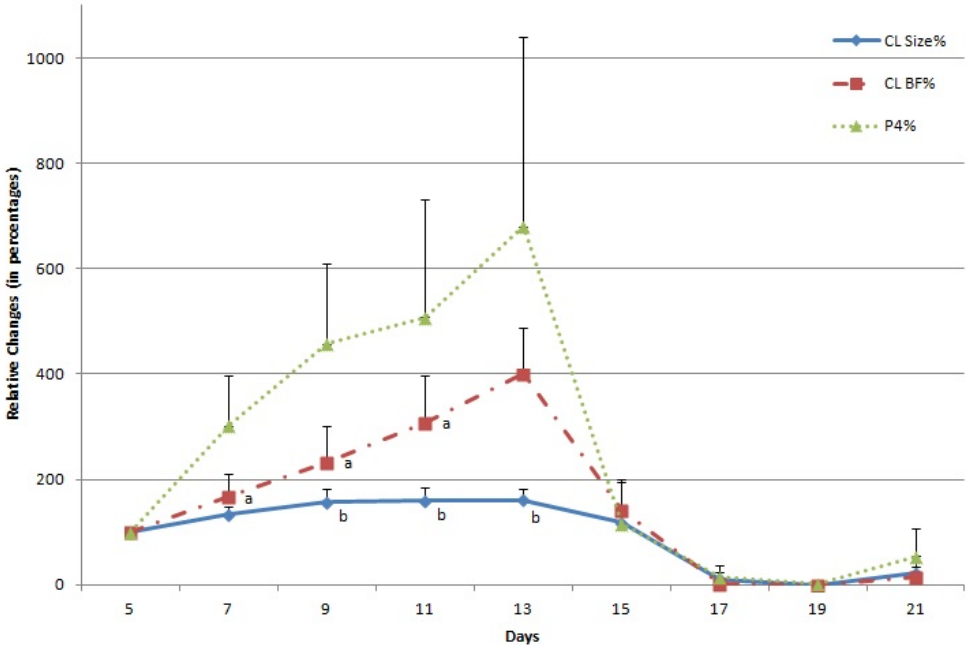

Figure 13: Mean ±SD of relative changes in blood flow (in percentages), mean ±SD of relative changes in progesterone levels (in percentages) and mean ±SD of relative changes in the diameter of corpus luteum (in percentages) during the experiment. Letters 'a' indicate significant differences ($p<0.05$) in relative changes between luteal blood flow and progesterone levels, letters 'b' indicate significant ($p<0.05$) differences in relative changes between luteal blood flow and diameter of the corpus luteum.

Discussion

One of the aims of this experiment is to evaluate the practical use of the Doppler technology. To achieve this goal the blood flow to the ovaries (corpus luteum and dominant follicles especially) during the estrus cycle are measured. The increase of blood flow during the luteal growth is correlated to the progesterone levels as well as the luteal size. Correlations between these measurements are also found by previous studies (Herzog et al, 2010; Herzog and Bollwein, 2007, Acosta et al, 2003).

The size of the corpus luteum is used for the differentiation between functional and non-functional corpora lutea, with a cut-off value of 4,5cm2 (Herzog et al, 2010; Bicalho et al, 2008). These sizes correspond with the static phase of the corpus luteum. During the static phase the size of the corpus luteum increases less than the progesterone levels and the blood flow. There is no correlation found between progesterone levels and luteal size, the correlation between the progesterone levels and blood flow was r=.201 (r=.500 for relative changes) during this experiment. Herzog et al found a slightly higher correlation during the static phase (r=.33, P=0.02). This suggests that the luteal size is a less appropriate indicator for luteal function (conclusion Herzog et al, 2010).

The progesterone levels and blood flow are also correlated during the regression phase, in agreement with Herzog et al (2010). Overall the correlation between the progesterone levels and blood flow is r=.788 (r=.842 for the relative changes), while the correlation between progesterone and luteal size was r=.766 (r=.738 relatively).

The blood flow to the follicles is researched in other studies, such as Acosta et al (2005). They found that the same percentages of the largest and second largest follicles have detectable blood flow until deviation. This indicates that the blood flow does not determine the changes of a follicle to become dominant. After deviation the dominant maintains a high blood flow, while smaller follicles do not. The dominant follicles produce vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, which leads an increase in blood flow to the follicle (Acosta et al, 2005). During this study the largest follicles showed a blood flow which was correlated (r=.351) to follicle size, but the results were largely disseminated.

There may be mistakes made which explain the small correlation found in this experiment. For instance colored pixels may be counted which do not belong to the follicles. These colored pixels are due to other moving obstacles, such as moving of the probe. Moving of the probe however causes a distinctive haze on a large part of the screen. This pattern should be recognized when the images are processed offline. Blood flow in other tissues, such as vessels, or the hand of the examiner may also create of colored pixels. This should also be recognized afterwards, because the colored pixels are not placed directly next to de follicles. It may also be explained settings of the

sonographic machine; the settings proved to be efficient for the examination of the blood flow to the corpus luteum, since the results are similar to the other studies, but may not be sufficient for the evaluation of the follicles.

Acosta et al (2003) discovered that the blood flow would increase quickly after the LH surge (Acosta et al, 2003). This has not been observed during this experiment. This may be because the cows were examined daily during luteal regression phase and therefore the examinations were too far apart to see the short-term increase in blood flow. After ovulation the blood flow decreases to increase again with the development of the corpus luteum.

It is attempted in this study to make a combination between the blood flow to the follicles and the corpus luteum. A correlation between the blood flow to the follicles and the follicular size and between the blood flow to the corpus luteum and progesterone is found. This combination can be valuable. During the regression phase most mistakes can be made by using luteal size as measurement for the corpus luteum function. The corpus luteum decreases in size less rapidly compared to the blood flow and progesterone levels. Because the regression phase takes up about 20 percent of the total cycle the possibility of making an incorrect decision based on the luteal size is approximately one in five.

The blood flow to the corpus luteum and the progesterone levels decrease rapidly in two days (Herzog et al, 2010). Two days before the ovulation the deviation of follicles has already taken place (Senger, 2003), so the blood flow to the dominant follicle is high. During the growth phase of the corpus luteum, the deviation of the follicles has not taken place yet, so the blood flow in the follicles is similar in the largest and second largest follicles. This indicates that the combination of a small corpus luteum, a low progesterone level and a low blood flow to the corpus luteum, combined with a high blood flow to the dominant follicle occurs right before estrus. While a small corpus luteum, low progesterone level and low blood flow to the corpus luteum combined with a low or average blood flow to the largest and second largest follicles indicates met-estrus or the start of di-estrus. Further research is required to confirm this hypothesis.

The second aim of this study was to evaluate the practical use of the Color Doppler technique for the veterinarians in the field. The literature found many situations where the Doppler technique can be more indicative than rectal examines or normal sonographic techniques. Especially for veterinarians with limited experience on the subject, the Doppler technique can be very helpful. The determination between a luteinized or follicular cyst, the diagnoses of early pregnancy and the diagnoses of embryonic loss are more easily made using Color Doppler.The experiment shows that the ovarian blood flow can also be indicative for identifying the phase of estrus cycles and therefore contributes to efficient fertility counseling.

There are however also drawbacks on the technique. For one, the probe of the sonographic device has to be held very still to obtain a clear view of the

blood flow to the ovaries, and when the user does not have much experience, this can be challenging. The settings of the US-Doppler machine are very important for reliable results and have to be tested and standardized.

The experminet was perormed by a researcher with limited experience with sonographic imaging and Color Doppler technology, wich makes the results more useful for practitioners, because they are probably as experienced with the Doppler technology. This gives an opportunity to evalute the technology for the inexperienced veterinarians.

One has to realise there are also large absolute differences between cows. Therefore several examinations must be done to determine a phase. In the experiment only six cows were examined. The different results from these cows are used to simulate a model of the blood flow. The model can not accurately predict a value for any cow, but the results of only these six cows are already strongly correlated. Therefor the correlation can still be used in other cows and can be very indicative for practitioners. Herzog et al did found a cutoff value of >0.6 cm2 for the luteal blood flow during the static phase, corresponding with 35

Another disadvantage of the color doppler technology is the offline analysis of the amount of colored pixels in the area of interest. Because this quantitative analysis has to be done after the examination, there is always time between the examination of the cow and the moment of advising the owner. A more experienced vetenarian might be able to estimate the amount of colored pixels and recognize the different phases of the estrus cylce more easily (Herzog et al, 2010). The blood flow can give an immediate idea of luteal function and can be caluculated afterwards if needed.

Finally, the size of the equipment and the cost of procurement are relatively high and this can be drawback; however these hazards have been decreasing over the last few years (Herzog, 2007).

Conclusion

This study summarizes the benefits of transrectal Color Doppler technique used in cattle reproduction and evaluates the ovarian blood flow during the estrus cycle. The technique is non-invasive and requires no use of hormones or medicine. It provides additional information which can be used for fertility counseling. Physiologic and pathologic processes in the genitalia can be visualized and different aspects of it can be measured semi-quantitatively. However experience is needed to make the full use of its benefits and its performance are not widely known under practitioners. Since the costs and size of the equipment have decreased, the technique may be available for more practitioners and make its way to success.

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Attachment

Doppler graphics-Protocol

Obtain images

The sonographic machine must be turned on to start, be sure to use a outlet if possible to avoid failure due to electricity shortage. The patient number must be admitted at the tab PATIENT/ARCHIVE. The settings must be adjusted under the tab USER. The following settings may be applied;

Frequency: 5 MHz
PRF Color: 750 Hz
Smooth: 0
Power: Max
Wall filter: medium
Persistence: 3
Sensitivity: medium
Mas Threshold: 3
Colormap: 3
Baseline: 50%

Locate the dominant follicles; try to make the imaged as clear as possible, with the largest diameter possible. If the image is clear the frame can be frozen using FREEZE and saved using IMAGE. Make three different images of the follicle and then turn on the Doppler Color mode (COLOR). Try to make the image as clear as possible, with as many blue and red pixels as possible. Again freeze the frame three times when the images are clear and save the pictures using IMAGE. This process is repeated when the Corpus Luteum is in focus. Locate the CL, try to make the imaged as clear as possible, with the largest diameter possible. If the image is clear the frame can be frozen using FREEZE and saved using IMAGE. Make three different images of the follicle and then turn on the Doppler Color mode (COLOR). Try to make the image as clear as possible, with as many blue and red pixels as possible. Again freeze the frame three times when the images are clear and save the pictures using IMAGE. When the six pictures are saved, go to the PATIENT/ARCHIVE tab and close the exam using CLOSE EXAM. The pictures can be stored on an UBS stick using EXPORTS and again EXPORT.

Analyze data

Open the ImageJ program on the computer and insert het USB stick. Open the images using File, Open and Select. The picture will be visible and the size of the follicle or CL can be measured using either the wand or the oval.

When the follicle or CL is in the marked area the area size of the area can be calculated. Analyze and Measure will give a pop up-screen with the measurement of the area. Open a colored picture and adjust the settings. Image, Adjust and Threshold give a pop up-screen with the thresholds of the color in the image. Put the Threshold method on Default, the Threshold color on red and the Color space on HBS. Then adjust the HUE en Saturation using the image as guideline. The colored pixels should all become red, but het black and white of the image not. When these adjustments are made use Select to select the colored areas and Filtered to take out other background colors. Use Analyze and Analyze Particles and adjust the Show to Mask, select Display results, Clear results, Summarize, Add to Manager and Exclude on edges. Press OK and search for the area of interest (by number). The number under Area is the amount of pixels in the selected (colored) area.

Raw Data

Corpus luteum size (in cm diameter) in six cows

Day	16	1801	1887	6110	8383	9411	Mean
3	0			0	0	1,09	
4		1,71	1,09		1,62	1,8	1,555
5	1,94	1,77	1,71	1,64	1,94	2,78	1,963333
6	2,15	2	2,03	2,79	2,53	2,9	2,4
7	2,54	2,49			2,89	3,17	2,7725
9	3,61	2,8	2,36	2,98	3,14	3,3	3,031667
11	3,41	2,86	2,33	3,08	3,35	3,53	3,093333
13	3,33	2,93	2,55	2,89	3,42	3,52	3,106667
15	2,71	2,31	1,76	2,14	2,25	2,61	2,296667
16	2,21	1,5	1,32	1,05	1,93	2,08	1,681667
17			0	0	0	1,87	0,311667
19	0	0		0	0	0	0
21	0	0	1,09	0	1,34	0	0,405

Figure 14: Means of diameter of the corpus luteum (in cm), values are of six cows.

Day	16	1801	1887	6110	8383	9411	Mean
3							
4							
5	100	100	100	100	100	100	100
6	110,8247	112,9944	118,7135	170,122	130,4124	104,3165	124,5639
7	130,9278	140,678	0	0	148,9691	114,0288	133,6509
9	186,0825	158,1921	138,0117	181,7073	161,8557	118,705	157,4257
11	175,7732	161,5819	136,2573	187,8049	172,6804	126,9784	160,1794
13	171,6495	165,5367	149,1228	176,2195	176,2887	126,6187	160,906
15	139,6907	130,5085	102,924	130,4878	115,9794	93,88489	118,9125
16	113,9175	84,74576	77,19298	64,02439	99,48454	74,82014	85,69756
17	0	0	0	0	0	67,26619	11,21103
19	0	0	0	0	0	0	0
21	0	0	63,74269	0	69,07216	0	22,13581

Figure 15: Means of the relative changes in diameter of the corpus luteum (in percentages), values are of six cows.

Day	16	1801	1887	6110	8383	9411	Mean
3	0			0	0	695	
4		1395	438		742	3238	1453,25
5	2380	1418	1164	1167	1147	3152	1738
6	2496	1570	1555	1639	1528	3909	2116,167
7	3311	2872			2377	3784	3086
9	3457	4683	2565	3419	2583	5902	3768,167
11	4339	6056	4370	3836	2905	8958	5077,333
13	9339	5470	4622	6497	4440	8872	6540
15	4433	2935	958	1568	1821	2613	2388
16	1406	1083	142	309	666	786	732
17	0	0	0	0	0	484	80,66667
19	0	0	0	0	0		0
21	0	0	438	0	506	0	157,3333

Figure 16: Means of amount of colored pixels in the corpus luteum (in pixels), values are of six cows.

Day	16	1801	1887	6110	8383	9411	Mean
3			1164	0	0	695	
4		100	100		100	100	
5	100	100	100	100	100	100	100
6	104,8739	110,7193	133,5911	140,4456	133,2171	124,0165	124,4773
7	139,1176	202,5388			207,2363	120,0508	167,2358
9	145,2521	330,2539	220,3608	292,9734	225,1962	187,2462	233,5471
11	182,3109	427,0804	375,4296	328,7061	253,2694	284,2005	308,4995
13	392,395	385,7546	397,079	556,7266	387,0968	281,4721	400,0873
15	186,2605	206,9817	82,30241	134,3616	158,762	82,89975	141,928
16	59,07563	76,37518	12,19931	26,47815	58,06452	24,93655	42,85489
17	0	0	0	0	0	15,35533	2,559222
19	0	0	0	0	0	0	0
21	0	0	37,62887	0	44,11508	0	13,62399

Figure 17: Means of relative changes of the amount of colored pixels in the corpus luteum (in percentages), values are of six cows.

Day	16	1801	1887	6110	8383	9411	Mean
3		0,1	0,1	0,1	0,4	0,7	0,28
5	0,9	0,3	1,2	0,8	1,1	1,5	0,966667
7	3,3	1,4	2,9	2,1	2,8	3,3	2,633333
9	4,2	2,1	4,6	4,5	3,9	4,2	3,916667
11	4,8	2,8	5,7	3,1	4,6	4,5	4,25
13	5,3	3,9	7,1	7	4,2	5,1	5,433333
15	1,5	0,5	2,8	0,4	0,3	0,8	1,05
17	0,1	0,1	0,2	0,1	0,1	0,1	0,116667
19	0	0	0,1	0	0,1	0	0,033333
21	0,1	0	1,2	0,8	1,2	0,1	0,566667

Figure 18: Levels of plasma progesterone (in ng/mL), values are of six cows.

Day	16	1801	1887	6110	8383	9411	Mean
3		0,1	0,1	0,1	0,4	0,7	0,28
5	0,9	0,3	1,2	0,8	1,1	1,5	0,966667
7	3,3	1,4	2,9	2,1	2,8	3,3	2,633333
9	4,2	2,1	4,6	4,5	3,9	4,2	3,916667
11	4,8	2,8	5,7	3,1	4,6	4,5	4,25
13	5,3	3,9	7,1	7	4,2	5,1	5,433333
15	1,5	0,5	2,8	0,4	0,3	0,8	1,05
17	0,1	0,1	0,2	0,1	0,1	0,1	0,116667
19	0	0	0,1	0	0,1	0	0,033333
21	0,1	0	1,2	0,8	1,2	0,1	0,566667

Figure 19: Relative changes in plasma progesterone levels (in percentages), values of six cows.

Statistic Analysis

		FSize	FBF
FSize	Pearson Correlation	1	,351**
	Sig. (2-tailed)		,005
	N	62	62
FBF	Pearson Correlation	,351**	1
	Sig. (2-tailed)	,005	
	N	62	62

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 20: Correlation between Follicular size and colored pixels

		CLSize	CLFB	CLFBP	P4
CLSize	Pearson Correlation	1	,818**	,784**	,766**
	Sig. (2-tailed)		,000	,000	,000
	N	52	50	52	52
CLFB	Pearson Correlation	,818**	1	,836**	,788**
	Sig. (2-tailed)	,000		,000	,000
	N	50	50	50	50
CLFBP	Pearson Correlation	,784**	,836**	1	,841**
	Sig. (2-tailed)	,000	,000		,000
	N	52	50	52	52
P4	Pearson Correlation	,766**	,788**	,841**	1
	Sig. (2-tailed)	,000	,000	,000	
	N	52	50	52	52

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 21: Correlations between corpus luteum size, corpus lutem colored pixels and progesterone levels, during the complete cycle

		CLSize	CLFB	CLFBP	P4
CLSize	Pearson Correlation	1	,928**	,671*	,747**
	Sig. (2-tailed)		,000	,034	,008
	N	14	14	10	11
CLFB	Pearson Correlation	,928**	1	,533	,710*
	Sig. (2-tailed)	,000		,113	,014
	N	14	14	10	11
CLFBP	Pearson Correlation	,671*	,533	1	,441
	Sig. (2-tailed)	,034	,113		,202
	N	10	10	10	10
P4	Pearson Correlation	,747**	,710*	,441	1
	Sig. (2-tailed)	,008	,014	,202	
	N	11	11	10	11

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Figure 22: Correlations between corpus luteum size, corpus lutem colored pixels and progesterone levels, during the luteal growth phase.

		CLSize	CLFB	CLFBP	P4
CLSize	Pearson Correlation	1	,344	-,391	-,172
	Sig. (2-tailed)		,162	,109	,494
	N	18	18	18	18
CLFB	Pearson Correlation	,344	1	,349	,201
	Sig. (2-tailed)	,162		,156	,425
	N	18	18	18	18
CLFBP	Pearson Correlation	-,391	,349	1	,318
	Sig. (2-tailed)	,109	,156		,199
	N	18	18	18	18
P4	Pearson Correlation	-,172	,201	,318	1
	Sig. (2-tailed)	,494	,425	,199	
	N	18	18	18	18

Figure 23: Correlations between corpus luteum size, corpus lutem colored pixels and progesterone levels, during the static phase

		CLSize	CLFB	CLFBP	P4
CLSize	Pearson Correlation	1	,875**	,883**	,555**
	Sig. (2-tailed)		,000	,000	,005
	N	24	22	24	24
CLFB	Pearson Correlation	,875**	1	,917**	,441*
	Sig. (2-tailed)	,000		,000	,040
	N	22	22	22	22
CLFBP	Pearson Correlation	,883**	,917**	1	,456*
	Sig. (2-tailed)	,000	,000		,025
	N	24	22	24	24
P4	Pearson Correlation	,555**	,441*	,456*	1
	Sig. (2-tailed)	,005	,040	,025	
	N	24	22	24	24

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Figure 24: Correlations between corpus luteum size, corpus lutem colored pixels and progesterone levels, during the luteal regression phase

		CLSize	CLBF	P4
CLSize	Pearson Correlation	1	,816**	,735**
	Sig. (2-tailed)		,000	,000
	N	64	64	52
CLBF	Pearson Correlation	,816**	1	,818**
	Sig. (2-tailed)	,000		,000
	N	64	64	52
P4	Pearson Correlation	,735**	,818**	1
	Sig. (2-tailed)	,000	,000	
	N	52	52	52

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 25: Correlations between the relative changes in corpus luteum size, corpus lutem colored pixels and progesterone levels, during the complete cycle

		CLSize	CLBF	P4
CLSize	Pearson Correlation	1	,765**	,872**
	Sig. (2-tailed)		,001	,001
	N	16	16	10
CLBF	Pearson Correlation	,765**	1	,830**
	Sig. (2-tailed)	,001		,003
	N	16	16	10
P4	Pearson Correlation	,872**	,830**	1
	Sig. (2-tailed)	,001	,003	
	N	10	10	10

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 26: Correlations between the relative changes in corpus luteum size, corpus lutem colored pixels and progesterone levels, during the lutal growth phase

		CLSize	CLBF	P4
CLSize	Pearson Correlation	1	,159	,272
	Sig. (2-tailed)		,530	,275
	N	18	18	18
CLBF	Pearson Correlation	,159	1	,378
	Sig. (2-tailed)	,530		,121
	N	18	18	18
P4	Pearson Correlation	,272	,378	1
	Sig. (2-tailed)	,275	,121	
	N	18	18	18

Figure 27: Correlations between the relative changes in corpus luteum size, corpus lutem colored pixels and progesterone levels, during the static phase

		CLSize	CLBF	P4
CLSize	Pearson Correlation	1	,862**	,713**
	Sig. (2-tailed)		,000	,001
	N	24	24	18
CLBF	Pearson Correlation	,862**	1	,710**
	Sig. (2-tailed)	,000		,001
	N	24	24	18
P4	Pearson Correlation	,713**	,710**	1
	Sig. (2-tailed)	,001	,001	
	N	18	18	18

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 28: Correlations between the relative changes in corpus luteum size, corpus lutem colored pixels and progesterone levels, during the regression phase

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	CLSize - CLBF	-49,12119	43,70600	19,54592	-103,38936	5,14698	-2,513	4	,066
Pair 2	P4 - CLBF	141,69129	97,46071	43,58576	20,67783	262,70474	3,251	4	,031

Figure 29: Paired Sample t-test between luteal blood flow and progesterone levels or luteal size (in percentages) on Day 7

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	CLSize - CLBF	-81,06057	73,74540	30,10643	-158,45162	-3,66951	-2,692	5	,043
Pair 2	P4 - CLBF	228,94413	128,42882	52,43085	94,16635	363,72191	4,367	5	,007

Figure 30: Paired Sample t-test between luteal blood flow and progesterone levels or luteal size (in percentages) on Day 9

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	CLSize - CLBF	-148,32012	96,75631	39,50060	-249,85964	-46,78061	-3,755	5	,013
Pair 2	P4 - CLBF	199,39163	188,23069	76,84486	1,85564	396,92763	2,595	5	,049

Figure 31: Paired Sample t-test between luteal blood flow and progesterone levels or luteal size (in percentages) on Day 11

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	CLSize - CLBF	-239,18137	75,71753	30,91155	-318,64204	-159,72069	-7,738	5	,001
Pair 2	P4 - CLBF	279,47491	378,35159	154,46139	-117,58074	676,53056	1,809	5	,130

Figure 32: Paired Sample t-test between luteal blood flow and progesterone levels or luteal size (in percentages) on Day 13

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	CLSize - CLBF	-23,01544	38,02607	15,52408	-62,92136	16,89047	-1,483	5	,198
Pair 2	P4 - CLBF	-25,71632	94,89314	38,73996	-125,30056	73,86792	-,664	5	,536

Figure 33: Paired Sample t-test between luteal blood flow and progesterone levels or luteal size (in percentages) on Day 15

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	CLSize - CLBF	8,65181	21,19252	8,65181	-13,58837	30,89199	1,000	5	,363
Pair 2	P4 - CLBF	13,59819	13,50853	5,51484	-,57815	27,77452	2,466	5	,057

Figure 34: Paired Sample t-test between luteal blood flow and progesterone levels or luteal size (in percentages) on Day 17

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 2	P4 - CLBF	4,86111	5,53817	2,26095	-,95084	10,67306	2,150	5	,084

Figure 35: Paired Sample t-test between luteal blood flow and progesterone levels or luteal size (in percentages) on Day 19

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	CLSize - CLBF	8,51182	13,19152	5,38542	-5,33184	22,35547	1,581	5	,175
Pair 2	P4 - CLBF	39,33897	39,73879	16,22329	-2,36433	81,04227	2,425	5	,060

Figure 36: Paired Sample t-test between luteal blood flow and progesterone levels or luteal size (in percentages) on Day 21

Images

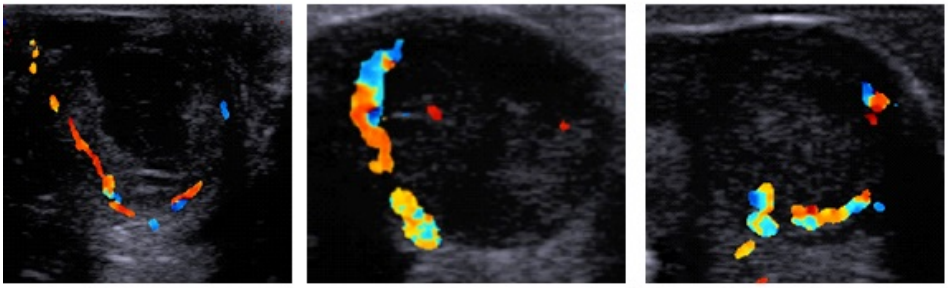

Figure 37: Examples of images of the blood flow in the corpus luteum in the growth phase

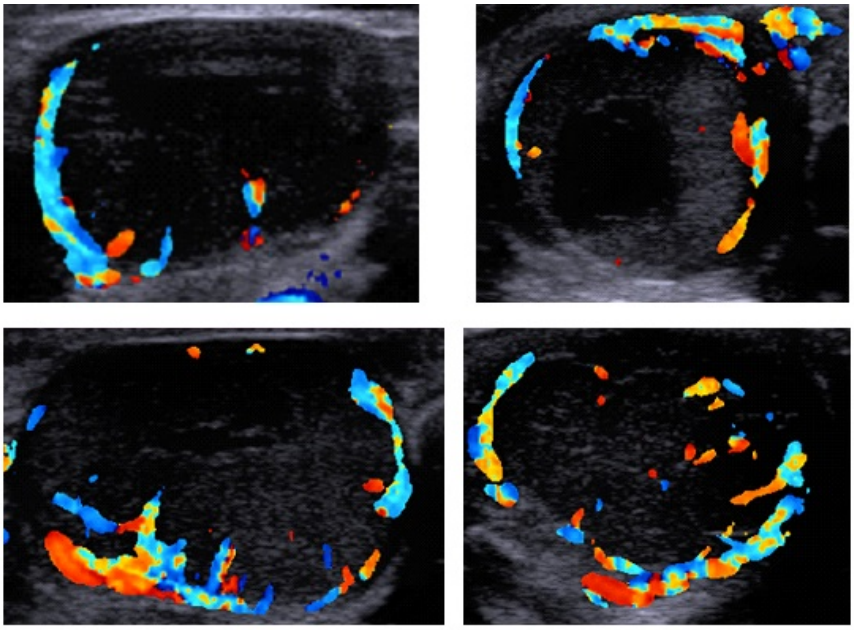

Figure 38: Examples of images of the blood flow in the corpus luteum in the static phase

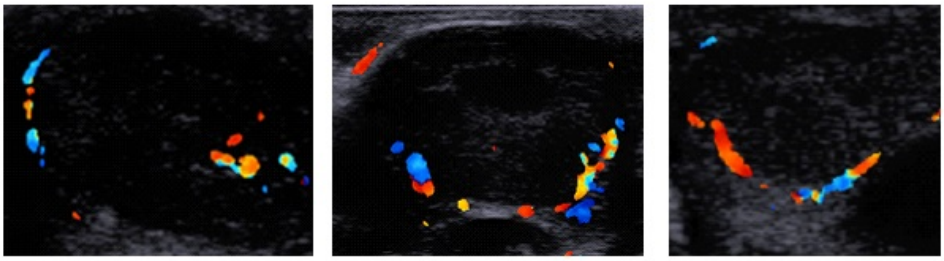

Figure 39: Examples of images of the blood flow in the corpus luteum in the regression phase

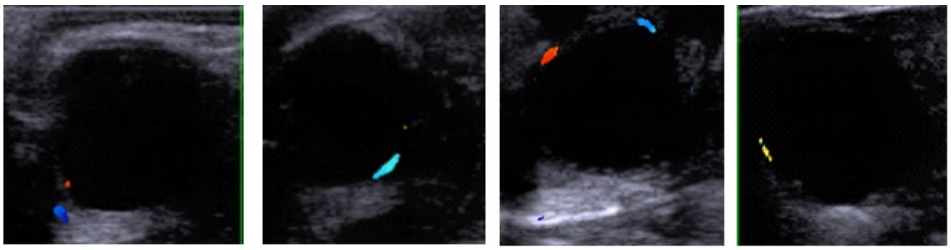

Figure 40: Examples of images of the blood flow to the follicle

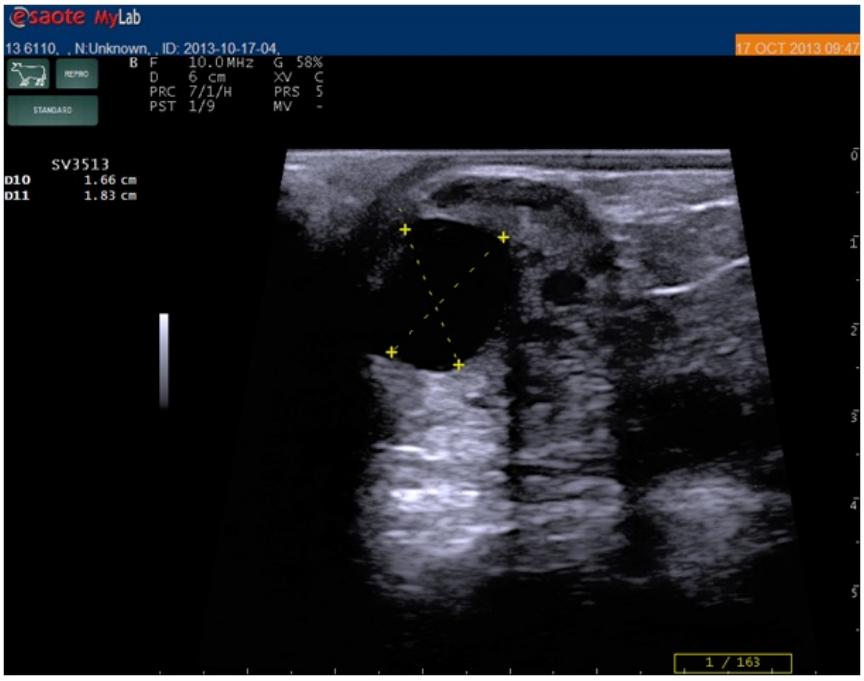

Figure 41: Example of measurements of the follicle

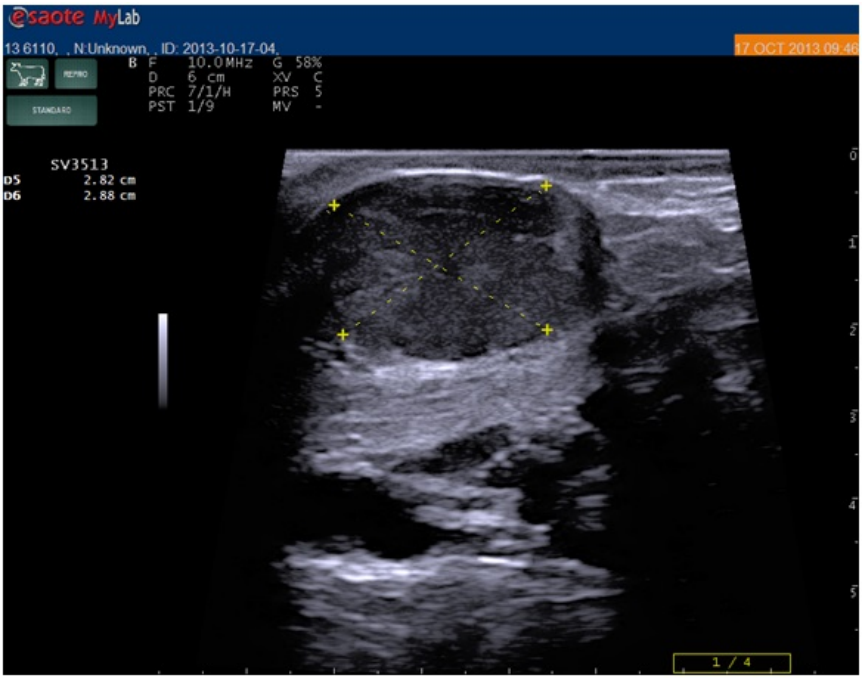

Figure 42: Example of measurement of the corpus luteum

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