UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

EQUINE CORPUS LUTEUM VASCULAR EVALUATION BY POWER-DOPPLER ULTRASOUND

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Abstract

The Doppler ultrasound is an emerging technology that has the potential to increase diagnostic capabilities of equine veterinarians. This technology is based on Doppler-shift frequencies, wherein the ultrasound frequency depends on the movements of red cells inside the vessels.

Corpus luteum (CL) function is dependent on blood supply, which not only provides steroid precursors but also releases progesterone into the systemic circulation. A more sensitive Doppler technique, the Power Doppler mode, was applied to evaluate a possible relationship between CL characteristics and plasma progesterone (P4) concentration. For this purpose, nine (n=9) mares were followed during the early breeding season. Corpus luteum diameter, area, and volume were assessed with B-mode grey ultrasound and vascularisation was evaluated using power Doppler and plasma P4 determination was performed by radioimmunoassay (RIA).

In this study, in disagreement with some previous reports, no relationship was found between the CL cross sectional diameters, areas and volumes with plasma P4 concentrations. The relationship between CL vascularised areas and pixels in the power Doppler images was visually assessed by two trained veterinarians. No colour intensity differences among samples was observed. Therefore, it is unlikely that a relationship between plasma P4 concentration and vascularisation exists in the equine CL.

Key words: Equine, *Corpus Luteum*, vascularisation, power-Doppler sonography, P4;

Resumo

A ecografia com recurso Doppler é uma tecnologia emergente que tem demonstrado ter potencial para melhorar as capacidades de diagnóstico dos veterinários de equinos. Esta tecnologia baseia-se nos príncipios do Doppler, onde as frequências de ultra-som estão dependentes do movimento dos eritrócitos dentro dos vasos sanguíneos.
A função do Corpo Lúteo (CL) está dependente do aporte sanguíneo, o qual fornece não só percursores esteroides mas também permite libertação de progesterona (P4) na circulação sistémica. Desta forma uma técnica Doppler mais sensível, o modo Power Doppler, foi utilizada para avaliar uma possível relação entre as características do Corpo lúteo e a concentração da progesterona plasmática. Para este estudo nove éguas (n=9) foram seguidas durante o início da estação reprodutiva. O diâmetro, área e volume do Corpo Lúteo foram então obtidos através do modo B, bem como a sua vascularização através do modo Power Doppler.As análises da concentração plasmática de P4 foram feitas com recurso ao rádio-immuno-ensaio (RIA).
No presente estudo, e contrariamente a estudos prévios, nenhuma relação foi encontrada entre o diâmetro, área e volume dos CL avaliados e a concentração de P4 circulante. Uma possível relação entre as áreas vasculares dos CL e dos píxeis obtidos nas imagens power Doppler foi visualmente avaliada. Porem nenhuma diferença na intensidade nos padrões vasculares foi observada, o que poderá indicar que não existe uma relação directa entre a concentração de P4 e a vascularização do CL.

Palavras-chave: Equinos, Corpo Lúteo, vascularisação, ultra-sonografia power-Doppler, P4;

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Previous note

During the last year in the Integrated Masters Degree of the Veterinary Medicine Faculty, it has been required to do a thesis based on the students final practical training. This practical period is of extreme importance, since the students acquire more practical experience and contacts with the extramural reality.

In this case, the author joined an Erasmus student program for 4 months (September-December 2007), which took place at the Large Animals Clinic Hospital from the faculty of Ghent- Belgium. Upon students arrival they are integrated in a scheme of rotation among departments of that hospital. Thus, the training included two weeks at the Reception department, two weeks at the Surgery department, two weeks at Internal Medicine, two weeks in Reproduction and Obstetrics, and four weeks in the Ambulatory clinic department.

At the Reception department, in most cases, lameness evaluations were performed and some neurological disorders were also diagnosed. Usually, the lameness cases were of difficult diagnosis and other veterinarians working outside the faculty referred them. The imagiology department functioned as a support of this department. In the Surgery department several surgeries were followed, which comprised, arthroscopies, orthopaedic surgeries, colic surgeries and others. At the Internal Medicine department the most of the cases followed were colics, but other gastrointestinal, respiratory and disorders of other systems were observed. There was a close relationship between that department and Surgery, since some of the colic cases were conducted at the surgery room. At the ambulatory clinic the most frequent cases were cattle clinic, where the caesarean sections were very prevalent. At the Reproduction department the students performed daily practices of rectal palpation and ultrasound scanning on cows and mares. Some cases of gemelar pregnancy reduction were followed. During the stay at the Reproduction and Obstetrics department it had been proposed to the author the subject of this thesis. Therefore, the practical part of this work was carried out at that department with the supervision of the assistants of the department.

Another part of the training was performed for five months (February-July 2008) at the Reproduction Department of the University of São Paulo, Brazil. During this period, stallion reproductive assessment was done, which included semen collection and evaluation. Other laboratory routines were followed such as the use of fluorescent probes for semen evaluation. Outside the faculty, the routine of two private reproduction centers was also followed. At those centers semen collection, mare breeding management, artificial insemination, embryo transfer and some neonatology cares were performed by the author.

Introduction

In the last decades the interest in horse breeding has increased. With the raise in the economical value of some horses, which are used for sport shows or only for amateur purposes, the assistance on their reproduction has become very important in the equine practice. Most of the reproduction interventions in horses are reserved to veterinary practice, and in the most of the cases they work with high quality animals, which represent an important genetical patrimony that has to be preserved. Some owners are willing to invest large amounts of money to get progenies from those animals, increasing the responsibility of the veterinary, which needs to apply the most recent technologies to answer the demands of the market.

During the last years we have witnessed an explosion in new reproductive technologies. Some of those technologies have already become routine of the breeding industry, while others will be used more slowly in the breeding industry and some may never be used.

Nowadays, assisted reproduction techniques of potential clinical use in the horse include embryo transfer (ET), oocyte transfer (OT), gamete intrafallopian transfer (GIFT), *in vitro* fertilization (IVF), intracytoplasmatic sperm injection (ICSI) and nuclear transfer (NT). However, only the embryo transfer is currently performed in the horse practice (Hinrich & Choi, 2005).

How quickly these technologies are accepted and utilized in the equine breeding industry depends upon the success of the technology, the attitude of the breeders and veterinarians, and the cost compared with the benefit of the technology.

Since the introduction of ultrasonography to the equine veterinary field in the early 1980's, its use as a diagnostic tool has expanded dramatically. Initially it was strictly used for pregnancy detection. However, nowadays, other applications in reproduction include: monitoring follicular changes and predicting ovulation; confirmation of ovulation and evaluation of the corpus luteum morphology, estimating the stage of the oestrous cycle, diagnosing ovarian irregularities and pathology; detection of twins and embryo reduction, determining embryonic death, evaluation of uterine pathology and evaluation of the testes and accessory glands in stallions (Squires, McKinnon, Shideler, 1988).

Doppler ultrasound technique revolutionized the ultrasound diagnosis and it is commonly used on echocardiography, abdominal exam, and depiction of some neoplasia based on characteristic vascular patterns. This technique is frequently used on assisted reproduction programs since ultrasound parameters of the endometrium and the evaluation of uterine and endometrial blood flow has long been considered as implantation markers in *in vitro* fertilization (IVF) and embryo transfer protocols in women (Borini *et al*., 2004; Miyazaki *et al.*,

1998). In the equine reproduction some applications of these mode are already available (Ginther & Utt, 2004).

The Corpus luteum (CL) is a transient endocrine gland, which develops from the follicular cells that remain after ovulation. This gland is required for the establishment and maintenance of pregnancy, since it is the major source of progesterone during the early pregnancy (Webb, Woad, Armstrong, 2002). Luteal insuffiency has been pointed as a cause in embryonic loss in other species as cows, nevertheless, in the mare it is not clear if a deficiency in luteal development or progesterone production can result in reduced fertility (Allen, 2001).

It has been shown, in studies on ewes, that blood flow to the luteal ovary increased during diestrus and then decreased dramatically at the end of the luteal phase; the decrease in the volume of luteal capillaries coincided with the decrease in blood flow to the CL (Knickerbocker, Wiltbank, Niswender, 1988).

A correlation between cyclic changes of the luteal blood flow and progesterone levels on plasma, have been found, using invasive methods, in other species (Brown, Emery, Mattner, 1980). Relationships between the morphoechogenicity and progesterone concentrations have already been made with conventional gray-scale ultrasound in heifers (Kastelic, Bergfelt, Ginther, 1990) and in mares (Bergfelt & Ginther, 1996; Arruda *et al.*, 2001).

The power Doppler is a more sensitive display to evaluate the small vessel perfusion (Rubin *et al.*, 1994), which characterises the CL vasculature. For several years, colour and power Doppler sonography have been used in human medicine to examine blood circulation of the CL, establishing a possible relationship between an insufficient blood perfusion of the CL and luteal phase impairment in women (Miyazaki *et al.*, 1998). Recently the power Doppler display has been applied for the CL evaluation in the mare (Bollwein, Mayer, Weber, Stolla, 2001) and its relationship with progesterone production has already been evaluated (Ginther, Gastal, Gastal, Utt, Beg, 2007).

The aims of this study were to evaluate a possible relation between: a) the measurements of luteal tissue: diameter, area and volume; b) the power Doppler ultrasound characteristics of luteal tissue in mares and the progesterone production of that luteal tissue.

Literature review

1.1 Ultrasound

Ultrasonography was introduced in the equine veterinary field in the early 1980's (Squires, McKinnon, Shideler, 1988). Some authors consider that gray-scale diagnostic ultrasonography is the most profound technological advance in the field of large-animal research and clinical reproduction since the introduction of the transrectal palpation and radioimmunoassay of circulating hormones (Ginther, 1986).

1.1.1 Grey scale ultrasound

In grey-scale ultrasound the amplitude of the returning echoes is displayed in form of grey images of variable brightness, which are determined by the manufacturer and cannot be changed by the operator (Reef, 1998). A lower grey scale occurs when penetration depth is increased. If available, a larger grey scale to assign the reflected ultrasound waves, it will conduce to a better tissue characterization of the image (Kremkau, 1993).

1.1.2 B Mode - Static and Real Time

The brightness mode, B-mode, is a two-dimension (2D) display of the returning echoes. The amplitude of the returning echo is converted into a dot with a characteristic brightness representing that returning echo (Reef, 1998), the brighter the dot, the stronger the corresponding echo. In this mode the location of the dot corresponds to the location of the echo reflector within the tissue cross-section (Reef, 1998). The B mode ultrasonography produces an image that corresponds to a cross section of the tissue within the plane scanned. This cross-section may be formed in the form of a single frozen image (static B mode), or be formed by the sum of numerous acquired frames to be displayed within one second (real time B mode).

Some post-producing methods may enhance the quality of the information that is obtained by ultrasound (US). For example, frame averaging, is a method that can improve ultrasound image resolution, performing by temporary storage of a part of the previous image, and to which is added a new image. For static structures, frame averaging can add additional echoes and increase the line density, thus improving image resolution. Other post- image producing processes can change the image brightness since the image is stored in memory (Reef, 1998).

Write magnification is a pre-processing function in which the image is magnified. However in this method the pixel size remains small. In contrast in read magnification, the image is magnified only after it has been frozen or stored in memory. The latter will result in a magnification of the pixels (Reef, 1998).

The frame rate is the number of frames displayed per second. The density of the obtained image is highest when a low frame rate is used, thus producing a higher-quality image. However, high frame rates are needed to visualize motion in real time, with the highest frame rates being used for structures that are moving very rapidly. For example in ultrasound of highly moving structures, such as echocardiography, frame rates must be high. Unfortunately to obtain this high frame rate some detail in image resolution will be lost (Reef, 1991). In contrast for scanning static structures, a low frame rate will be ideal to obtain the highest possible - image resolution. Frame rate can also decrease in other structures such as when tissue penetration increases, when wide angles for image visualization are used, or when the number of focus points increases (Kremkau, 1993).

1.1.3 Doppler

Thirty years ago, the use of Doppler ultrasound was limited to the vascular laboratory and mainly to evaluate the carotid arteries (Rubens, Bhatt, Nedelka, Cullinan, 2006). The basic physical principles that made Doppler blood flow measurement possible have been understood since World War II. Since then physicists and engineers have continued to improve Doppler ultrasound instrumentation, allowing clinicians to make accurate diagnoses of cardiovascular diseases (Taylor & Holland, 1990).

Nowadays, Doppler US is used in all areas of diagnostic US imaging, and extensively in abdominal, pelvic and obstetric imaging (Rubens *et al.*, 2006). Although Doppler is commonly used to measure blood flow, any tissue or fluid motion may generate a Doppler signal (Rubens *et al.*, 2006).

The Doppler-shift frequency, or Doppler frequency, is defined as the difference between the frequency of the transmitted ultrasound waves and the frequency of the received echoes (Ginther & Utt, 2004). The greatest difference or strongest signal is achieved when the motion is parallel to the US beam. In opposite, no signal is generated when the motion is perpendicular to it (Rubens at al, 2006).

1.1.4 Physical principles

In 1842, Christian Doppler published the theory that the frequency of a wave changes between a moving wave source and target. Based on the shift of the sound frequency of a moving train, a stationary listener can decide whether a train is approaching or receding. With an approaching train the sound becomes louder as it approaches (positive Doppler shift) (Lang, 2006).

The shift frequency, that characterises the Doppler effect, can be obtained by the following equation 1:

$$f_D = 2f_0 \upsilon \cos.\theta / c$$

where f_D is the Doppler frequency shift, f_0 is the incident frequency, υ is the flow velocity, c is the speed of sound in tissue, and θ is the angle between the ultrasound beam and the flow direction. The equation can only be applied when the frequency of an ultrasound echo from a moving reflector or scattered differs from the frequency of the original sound wave. The Doppler shift frequency (f_D) will depend on the velocity of the reflector and its direction, if and only we assume that the frequency and sound velocity are constant for that specific measurement (Taylor & Holland, 1990).
In Doppler ultrasound we can distinguish the spectral mode, which comprises the pulsed and continuous wave mode, and the colour mode, which includes the Power mode. For medical imaging two types of Doppler ultrasound are commonly used: pulsed-wave and continuous-wave Doppler. However Colour-flow Doppler is now extensively used for echocardiography and abdominal imaging. Power Doppler consists in a more recent development and is used mainly in abdominal or small-part imaging where low blood velocities are encountered (Lang, 2006).

1.1.5 Transducer

The appropriate selection of a transducer depends on the structure that will be evaluated, the depth of the area to be scanned to the transducer surface and on the acoustic tissue characteristics (Reef, 1998).

Transrectal transducers were one of the first US transducers to be used on Veterinary Medicine, to scan the female reproductive tract from the rectum. These transducers are, normally characterized by having a cigar shape and a beam originating at a 90 degrees angle from the long axis of the transducer (Reef, 1998).

Linear-array or convex-array transducers are most commonly used for transrectal Doppler imaging in horses. The linear-array transducer displays a rectangular image and is particularly useful for visualizing a large structure close to the transducer in one single image (Ginther & Utt, 2004). These transducers allow for the evaluation of the female and male inner reproductive tract, but also of the aorta, iliac arteries, bladder, urethra, cranial mesenteric artery, peri-rectal masses, and portions of the gastrointestinal tract (Reef, 1998). The most important disadvantage of these transducers is the fact that they have to be placed directly over the evaluated area, since the penetration depth is normally limited (Reef, 1998). The convex-array transducer produces a pie-shaped image (narrowest at the transducer) and may be smaller (finger-grip probe) and easier to manipulate and orientate within the confines of the rectum (Ginther & Utt, 2004).

1.1.6 Frequency

The frequency is the number of cycles or complete variations of the US beam per unit of time. It is expressed in mega-hertz (Reef, 1998). The frequency of ultrasound waves can be fixed (for single-frequency transducers) or controlled by the operator (in multi frequency transducers).

To a higher frequency corresponds a shorter wavelength. This represents the distance travelled by US during one cycle. A shorter wavelength also corresponds to an image with higher resolution (Reef, 1998).

Higher frequencies are much more sensitive to flow but cannot penetrate deep enough without suffering attenuation. As a consequence in superficial structures such as the testes, a frequency of 7 to 10 MHz will be ideal, whereas for deep abdominal structures, such as the hepatic arteries or portal vein, a 3 MHz or even a lower frequency will be needed (Rubens, 2006). A high frequency is more attenuated than the low-frequency sound wave, which limits the depth of tissue that US beam can penetrate (Kremkau, 1993).

1.1.7 Power

The acoustic power, output power, or drive voltage amplitude in some models, is the power or energy of US waves emitted from the transducer. This is a variable that can be controlled by the operator. Increasing the power will enable that weaker signals can be detected (Ginther & Utt, 2004). Increasing the power also increases the intensity of US energy delivered to tissues. However an optimal image quality is obtained when the lowest power

setting is used for the exam area, as the high-power settings result in more artifacts. (Reef, 1998)

1.1.8 Pulse Repetition Frequency

The interval between pulses is called pulse repetition frequency (PRF). This can be controlled by the operator, which can select the range of velocities to be sampled (Reef, 1998). A high PRF setting is used when the targeted vessels are near the transducer or when there is high blood flow. Conversely a low PRF setting is used when vessels are situated far from the transducer or the blood flow is slow (Ginther & Utt, 2004).

1.1.9 Doppler angle

Unlike grey scale US imaging, whereby the best image is obtained perpendicular to the US beam, in Doppler the strongest signals result when the motion is parallel to the beam. A Doppler angle of 90º will not display flow because no frequency shift is directed back towards the transducer (Rubens *et al.*, 2006). In fact as is depicted in the equation 1, when the ultrasound beam is at right angles to the vessel ($\cos 90^{o}=0$), the Doppler shift frequency is theoretically zero and no signal will be obtained (Taylor & Holland, 1990).

The larger the Doppler angle, the greater the correction that needs to be done and the greater the possibility of error. Therefore, Doppler beam angle must always be kept as low as possible. Ideally, it should be less than 60º and always less than 70º because the percentage of errors obtained by poor angle correlation can increase up to 20% to 30% with higher Doppler angles (Zweibel & Pellerito, 2005). When the transducer is aligned with vessel ($\cos 0^{o}=1$), the largest Doppler shift will be obtained and angle uncertainty has its least effect. However, there may be technical difficulties in obtaining signals at such low angles because of total reflection of sound waves at the vessel walls (Taylor and Holland, 1990).

1.1.10 Sample volume

The sample volume is the three-dimensional space from which the Doppler frequency shifts are measured. In colour or power Doppler it is the colour box, and in pulsed wave Doppler it is indicated by the cursor, which is placed within the vessel (Rubens *et al.*, 2006). In most systems, its dimensions are adjustable and depend on the focusing characteristics of the transducer and the number of cycles within the pulse, which determines the axial resolution (Taylor and Holland, 1990). Signals can be sampled and displayed from unwanted areas of a vessel or even from unwanted vessels such as adjacent arteries or veins. The ideal sample volume size for performing a routine survey of a vessel consists in about two thirds of the vessel width, if positioned in the center of the vessel width (Zweibel & Pellerito, 2005) and

excluding the unwanted clutter from near the vessel walls as much as possible (Merrit, 2004).

1.1.11 Pulsed-wave Doppler

The principles of pulsed-wave Doppler are similar to B-mode imaging, where sound is transmitted in short pulses and received by the same crystal during the time interval between emissions of pulses (Lang, 2006). By setting the sample volume in a vessel at a specific depth, the returning echoes from this vessel will arrive at a specific time interval. This time interval corresponds to the depth of the vessel (range gating), allowing the blood flow of a specific vessel to be measured. In this method, the location of a flow pattern can be precisely determined. The size of the gate is usually called the sample volume and can be adapted accordingly to vessel size. In this technique only one crystal is used to transmit and receive sound and so the depth of pulsed-wave Doppler, as the measurable obtained velocities, are limited. In order to measure blood flow velocity correctly, the pulse repetition frequency must be twice the highest frequency of returned echoes (which is known as the Nyquist limit). When the Nyquist limit is exceeded, an artifact known as aliasing occurs, making accurate interpretation of the flow velocity impossible. Simply stating aliasing occurs when the pulse repetition frequency is too low, when there is high flow velocity (e.g.: large vessels) or when the sample depth is too great. In these cases it will be necessary to change to continuous-wave Doppler ultrasound to obtain accurate measurements.

1.1.12 Continuous-wave Doppler

In this technique, the ultrasound is transmitted and received continuously. The transducer contains two different types of crystals, one that transmits and one that receives sound. The sound waves can therefore be received continuously and so continuous-wave Doppler can be used for evaluation of very high velocities (Lang, 2006).

1.1.13 Wall filters

Unwanted Doppler signals from large moving structures can often be avoided by consulting the US image obtained with duplex and colour Doppler systems, and by cutting-of the low frequency noises (Reef, 1998). However, Doppler shifted ultrasound echoes from vessel walls are more problematic, since they typically lie in the region of interest (Taylor and Holland, 1990).

The Doppler frequency shift can be detected from moving blood vessel walls and from the blood itself. The wall echoes have large amplitude, which causes a loud “wall thump” on the

audio Doppler output (Taylor and Holland, 1990). These signals have low frequency and can be cutted by using a higher threshold, obtaining a cleaner high-velocity blood-flow signal. However if the wall filter threshold is set too high, true blood flow can also be discarded from the display. Low velocity venous flow and the filter for venous Doppler should be kept at the lowest practical level, usually 50 to 100 Hz or less (Merrit; 2004)

1.1.14 Doppler gain

This setting controls the amplitude of the colour display in colour or power Doppler and spectral display in pulse Doppler mode (Rubens *et al.,* 2006). To adjust Doppler gain for colour imaging, the former should be turned up until the image of scattered isolated colour pixels can be seen overlying the grey-scale background. At this moment the gain should then be decreased until it disappears.

If the colour gain settings are set too low, flow will be present but not detected. If settings are set too high, colour or power signals may overwrite grey-scale clot. A machine setting related with the gain for colour and power Doppler is the colour-write priority, which determines whether a given pixel is written as a grey-scale value or as colour (Zweibel & Pellerito, 2005). If the grey-scale signal is above some threshold, the pixel remains grey. If the signal is below the threshold, the pixel is displayed as coloured. If the grey-scale gain is too high or the colour-write priority too low, some colour pixels may not be displayed as well (Rubens *et al.*, 2006)

1.1.15 Velocity scale

The velocity scale controls the range of frequencies that is displayed and it is a critical setting in colour and spectral Doppler imaging. If the scale is set too high, the dynamic range is too large and low velocity signals will be missed, particularly those obtained from low flow vessels. If the velocity scale is set too low, the dynamic range is too small to display the high-velocity signals accurately and the artefact of aliasing will occult (Rubens *et al.*, 2006). So this setting should be adjusted to the structure characteristics that have to be evaluated.

1.2 Doppler artifacts

Artifacts are common on colour Doppler images as well as in B-mode images (Ginther& Utt, 2004; Rubens *et al.*, 2006). The Doppler instrument is sensitive not only to the movement of red cells but also to extraneous movements or clutter as well, such as the case of tissues or animal movements. This will lead to *colour* or *flash artifacts*, which can be extensive and obscure the colour displays in the colour mode.

These artifacts can be grouped into three broad categories:

1. artifacts caused by technical limitations, including aliasing, improper angle with no flow, indeterminate Doppler angle, blooming, and partial volume artifacts;
2. artifacts caused by patient anatomy, including mirror image artifact, flash artifact, an pseudo flow;
3. artifacts caused by machine factors, including edge artifact and twinkle artifact.

1.2.1 Edge and Twinkle artifacts

The edge artifact may be generated by any echogenic surface, and it refers to the Doppler signal generated at the margin of a strong, smooth, specular reflector (Reef, 1998). This artifact is more commonly seen with Power Doppler US since a large dynamic range is used (Reef, 1998).

The twinkle artifact is defined as a colour Doppler signal that imitates flow behind a stationary reflecting interface such as a renal calculi or bladder calcification (Reef, 1998).

1.2.2 Aliasing

Aliasing is a common artifact of the Doppler spectrum. Basically, aliasing occurs when the velocity range of the scanned sample exceeds the scale available to display it. The maximum velocity scale is limited by the number of US pulses per second that can be transmitted and received by the transducer and depends on the transducer used. The PRF setting requires a scale that must be, at least, twice the frequency of the Doppler signal (Zagszebski, 2000). In practical, the display “wraps around” the scale and overwrites the existing data.

There are several solutions to avoid spectral aliasing. The first one includes to drop the baseline or to increase the velocity scale (PRF) and/ or to increase the available velocity range. If the scale is still inadequate, the Doppler frequency shift can be decreased by using a lower frequency or by increasing the Doppler angle (Taylor and Holland; 1990). Although increasing the PRF decreases aliasing, this will imply less ability to detect a slow flow. The opposite is also true: a low PRF results in better detection of slow-moving flow but also

increases the likelihood of aliasing (Ginther & Utt; 2004). Power Doppler, on the contrary, has no aliasing because it has no directional or velocity component (Rubens *et al.*, 2006).

1.2.3 Blooming artifact

This artifact is also known as “colour bleed”, because the colour spreads out from the vessel and “bleeds” beyond the wall into adjacent areas. This artifact is created because colour US image is actually constructed from two images superimposed, the colour and the grey-scale. Thus, depending on how the parameters are set, the colour portion of the image can extend beyond the grey-scale vessel margin (Nilsson, 2001). The unwanted result is that the subject to be scanned within the vessel can be “ written over” and obscured (Forsberg, Liu & Burns 1994).

B-flow, an alternative US-based blood flow detection method, can be useful when colour imaging is problematic (Weskott, 2000).

1.2.4 Directional ambiguity

Directional ambiguity, or indeterminate flow direction, is a term that refers to an artifact that occurs with spectral Doppler tracing where its waveform is displayed with nearly equal amplitude above and below the baseline, creating a mirror image pattern. This pattern results when the interrogating beam intercepts the vessel at a 90º angle (Pozniak, Zagzebski & Scanlan; 1992). This artifact is most commonly seen in small vessels, especially in those travelling in and out of the imaging plane (Rubens *et al.*, 2006), and it may be corrected by changing the angle of the interrogating beam.

1.2.5 Partial volume artifact

Partial volume artifact results from a slice thickness, which is not thin enough to be entirely included on the plane that has to be scanned. Echoes and Doppler signals can be obtained from objects, partly from within the slice and partly from outside of it (Goldstein & Madrazo; 1981). So, this artifact creates echoes that on the display appear like curved, fluid-filled structures such as anechoic vessels or ducts, which in reality are not existent (Kremkau & Taylor; 1986). These echoes can also mimic dependent particulate material, which in turn can be misinterpreted as thrombus, debris, or sludge. These echoes can appear within anechoic structures and Doppler signals are acquired in an area in which no vessels are perceived on grey-scale.

1.2.6 Pseudo flow

Pseudo flow is defined as presence of movement of fluid other than blood (Campbell, 2004). Pseudo flow can mimic real blood flow with colour and Power Doppler. The difference is that there are not any true existing vessels. Examples of pseudo flow include ascites, amniotic fluid and urine (Rubens *et al.*, 2006).

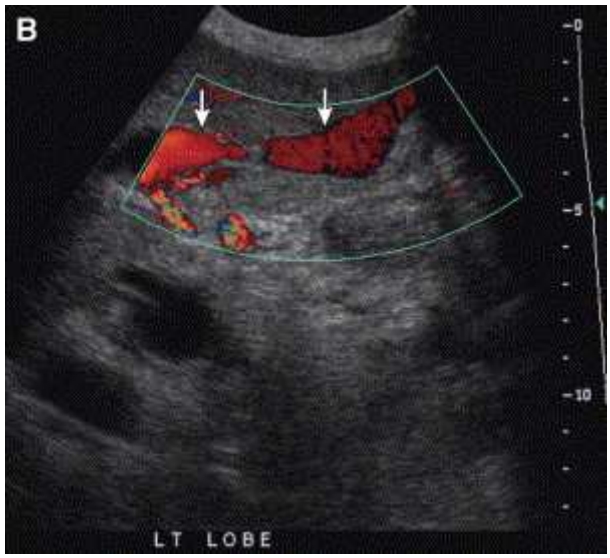


Fig 1- Pseudo flow in liver.

1.2.7 Flash artifact

Flash artifact is a sudden burst of random colour that fills the frame, obscuring the grey-scale image. This artifact can be caused by object motion or transducer motion (Campbell, 2004). Power Doppler is more susceptible to flash artifacts than colour flow Doppler because of the longer time required to build the image. This occurs because more frames are averaged together to create the image in power Doppler compared to standard colour Doppler (Zagzebski, 2000).

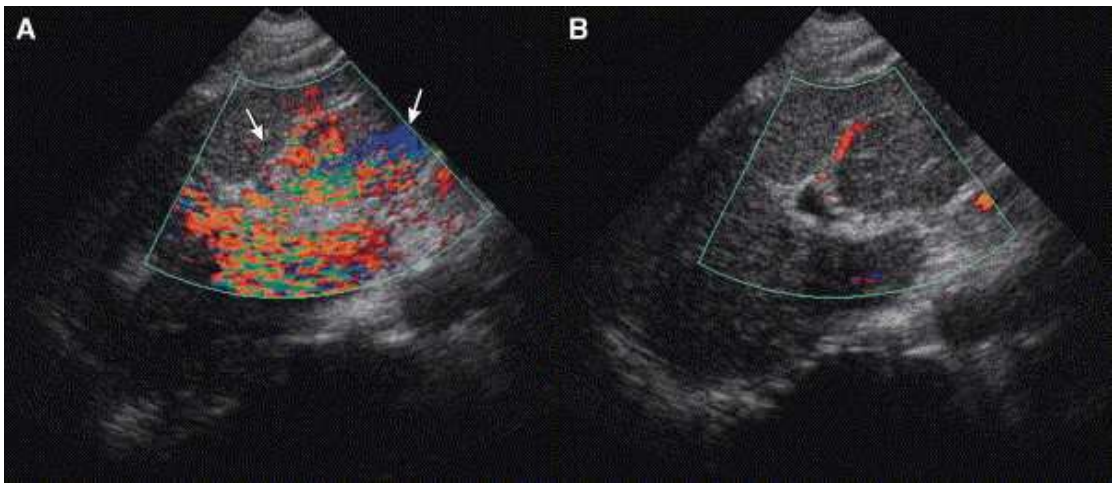


Fig 2 - Flash artifact. (A) Longitudinal CDUS through the left lobe of the liver with flash artifact, produced by respiratory motion. Longitudinal CDSU with no motion shows normal vascular flow with no artifact. (Adapted from Rubens *et al.*, 2006)

1.3 Power Doppler

Power Doppler is a colour Doppler mode in which an estimate of the total integrated Doppler power spectrum rather than just a single estimate of the mean frequency is displayed on the frame. This will give power Doppler an increased sensivity to low-flow states (Rubin, Bude, Carson, Bree & Adler, 1994; Reef, 1998). In this mode the shade and brightness of the colour signal represent the total energy of the Doppler signal (Rubin *et al.*, 1994).

The main advantage that power Doppler ultrasound has over normal colour Doppler is that increased gain settings can be employed (Rubin *et al.*, 1994).

In colour Doppler, noise appears in the form of white images and can result from a flow of any frequency shift. If the gain is set too high, the random background of noise quickly obscures the flow signal, and will then itself be displayed as flow. In power Doppler, because the noise consists in echoes of uniformly low power, it will be displayed as a background of uniform appearance. Consequently it is much easier to perceive the real flow in Power Doppler (Bude & Rubin, 1996).
Power Doppler Ultrasound is also less angle dependent than the colour Doppler US (Rubin *et al.*, 1994; Bude & Rubin, 1996). As the Power Doppler technique does not imply the need of any frequency, velocity or directional information, the aliasing artifact will not affect it (Bude & Rubin, 1996).

The Power Doppler technique increase the sensitivity for displaying blood flow in soft tissue, 3 to 5 times than conventional colour display for Doppler sonography (Rubin *et al.*, 1994; Amso, Watermeyer, O'Brien & D'Angelo 2001). This greater sensitivity also allows for the

evaluation of vessels with smaller diameters or slower flow than what is currently possible with conventional colour-flow images. It can also provide a better definition of tortuous vessels (Martinoli *et al.*, 1998)

However, power Doppler does have some disadvantages compared to colour Doppler. One disadvantage is the increased susceptibility of Power Doppler to display flash artifacts, which if excessive might preclude its use (Bude and Rubin, 1996; Rubens *et al.*, 2006). Another disadvantage is its lack of directional or velocity flow information (Bude and Rubin, 1996).

1.4 Clinical Applications – Human Medicine

Due to the increased sensitivity to depict the continuity of blood flow and the improved definition of intravascular edge, Power Doppler technique shows significant advantages over Colour Doppler (Martinolli *et al.*, 1998).

1.4.1 Depiction of vessel morphology

In large arteries, PD was shown to enhance luminal measurements accuracy in high-grade stenosis due to complex plaque composition and calcification, and to contain the ability to differentiate plaque surface morphology (Steinke, Meairs, Ries & Hennerici, 1996). In this area of investigation, intrinsic limitations of CD consist in its inability to visualize the residual lumen in high-grade stenosis, as well as the tendency to the noise to overwhelm the flow signal, which may result in an over or underestimation of stenosis (Steinke *et al.*, 1996).

Also, in venous system, the ability of PD in differentiating a slow versus an absent flow can improve diagnostic accuracy in detecting blood clots (Babcock, Patriquin, LaFortune & Dauzat, 1996). In abdominal veins, PD has also been shown to provide a better description of the cavernous transformation of the portal vein and to increases the delineation of floating thrombus (Martinoli *et al.*, 1998).

1.4.2 Tissue perfusion

The improved detection of tissue vasculature has some potential benefits especially in the fields where CD is not sensitive enough to detect clinically important, slow, low-volume flows in small vessels (Martinoli *et al.*, 1998). For most cases, PD will make the intraparenchymal vasculature visible as a subtle network of small discrete vessels.
Power Doppler has also been proposed as a mean to evaluate conditions that will alter blood flow distribution on the renal cortex, such as focal inflammatory regions, abscesses and

tumours. It can also be used in conditions, which result in a markedly decreased or absent renal cortical vascularity, and to detect small renal tumours (Bude, Rubin & Adler; 1994).

PD has also been used to evaluate testicular perfusion. The most important clinical condition where imaging testicular flow can be beneficial is to differentiate testicular torsion from epididymiditis-orchitis. It was shown that Colour Doppler is an efficient method in the evaluation of the acute inflamed scrotum. However, some investigators reported that intratesticular flow can be detected more sensitively with Power Doppler (Bader, Kammerhuber & Herneth; 1997).
Colour Doppler ultrasound studies have yielded new information on uterine blood circulation during the menstrual cycle in women (Kurjak *et al.*, 1995). Uterine arterial blood flow can now be used to predict a hostile uterine environment prior to embryo transfer (Steer *et al.*, 1992).

Avascular necrosis of the femoral head still remains a possible complication from treatment to hip dysplasia and specially when positional abduction restrains are used. Power Doppler enabled to depict vessels within the cartilaginous femoral head in some studies (Bearcrof, Berman, Robinson & Butler; 1996).

Although there is susceptibility of Power Doppler to display artifacts caused by some physiological movements, such as intestinal peristaltic contractions, it has been used with success to distinguish viable from non-viable bowels, in patients with focal thickening of the gut wall (Clautice-Engle, Jeffrey, Li, & Barth, 1996).

With its enhanced sensitivity, Power Doppler is also valuable for detecting increased blood flow in vessels that are dilated as a consequence of an inflammatory response. In fact, some studies reported that in these situations PD often shows a diffuse blush in acutely inflamed tissues (Stavros, Rapp & Thickman, 1995). In the literature, advantages in using Power Doppler have also been reported in acute cholecystitis (Uggowitzer *et al.*, 1997) and in inflammatory states of musculoskeletal tissues (Newman *et al.*, 1994).

The enhanced sensitivity to the detect low velocity blood flow vessels and to display with accuracy the delineation of tortuous and irregular vessels, makes Power Doppler a promising technique to image intratumoural vessels. If properly used PD can thereby, ameliorate Colour Doppler accuracy for predicting the likelihood of benign versus malignant nodules. In fact, by studying the nodular vascular pattern, many authors have shown that the resistive index (RI) (RI=[PSV-EDV]/PSV; PSV, peak systolic velocity; EDV, end diastolic velocity), values in malignant nodules were significantly higher than in benign nodules (Tamsel *et al.*, 2007).

1.5 Doppler Ultrasound in Equine Reproduction

Transrectal B-mode (grey scale) ultrasonography has revolutionized diagnosing and monitoring of biologic and pathologic reproductive events in horses and cattle, for both clinical and research areas (Ginther, 1995). In these species, B-mode is not only used to identify and measure structures, but also to assess physiologic status (Ginther & Utt, 2004).

Nowadays, Doppler ultrasound seems to be able to provide a major positive impact on the diagnosis and predictive capabilities of equine reproductive theriogenologists and research scientists. The extent of vascular perfusion of a structure can not only indicate the current status of the structure, but it also can provide indications for its future viability (Ginther & Utt, 2004).

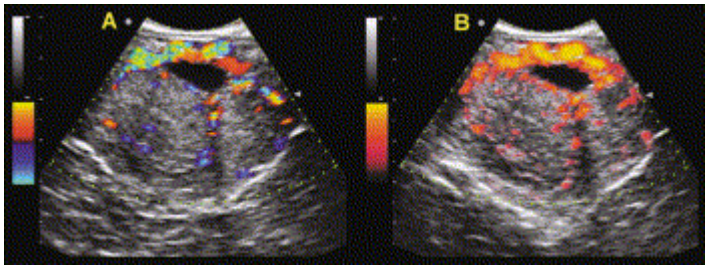


Fig 3- Images of 2 CL from a 48-day pregnant mare shown in colour-flow mode (A) and power-flow mode (B) from similar sections. (Adapted from Ginther & Utt; 2004)

1.5.1 Colour Doppler ultrasound evaluation of testicular blood flow in stallions

Colour Doppler ultrasound has become a method of choice to evaluate vasculature of various organs, including testes. In Human Medicine, this technique has been used to evaluate blood flow in the testicular artery and has also been applied for the diagnosis of testicular pathologies associated with altered blood flow, including spermatic cord torsion, testicular infarction or varicocoele (Aydos *et al.*, 1993; Pavlica & Barozzi, 2001; Sidhu, 1999; Sriprasad *et al.*, 2001).

Colour Doppler ultrasound appears to be useful in identifying early inflammatory or neoplastic changes of the testes and epididymes, as well as in evaluating other scrotal disorders in people (Herbener; 1996, Gorecka-Szyld; 1999).

In stallions, the resistive index (RI) of testicular arteries seems to be the most useful clinical measure of blood flow to and within the testis and epididymis. This parameter can suffer variation in inflammatory processes but also caused by aging (Jee *et al.*, 1997; Wielgos *et al.*, 1998). However, in one group of studied stallions, although age was not shown to be a

significant factor, older stallions had lower values of EDV- end diastolic velocity and greater values of RI than the middle age stallions (Pozor *&* McDonnell, 2004).

Colour Doppler ultrasound characterization of blood flow of the stallion testis is possible, and is becoming an useful tool for objective evaluation of the stallion testis, particularly in cases of scrotal disorders with different etiologies (Pozor & McDonnell, 2004). The presence of a turbulent blood flow was demonstrated in some severe cases of hydrocoele (Pozor & McDonnell, 2004). A characteristic vascularisation of testicular tumour in the stallion and abnormal course of large blood vessels within the testicular parenchyma after a trauma was also seen (Pozor & McDonnell; 2004).

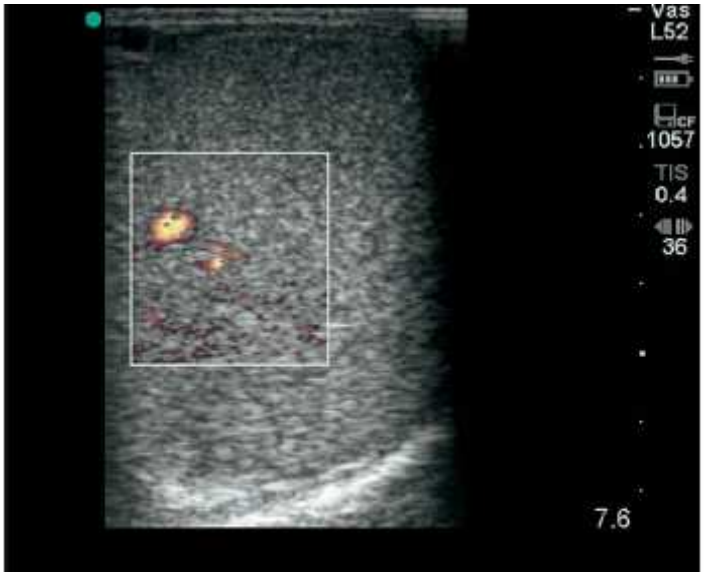


Fig 4 - Power Doppler ultrasound image of central vein and small intratesticular vessels

1.5.2 Colour Doppler evaluation of the uterine artery in mares

In the early luteal phase, mares present a low Resistive Index (RI), indicating a decreased impedance to blood flow in distal vasculature and consequent increased uterine perfusion. It seems logical that uterine blood supply would be higher in the early luteal phase, which corresponds to the time of entry of the embryo into the uterus. In fact uterine blood supply is important for embryo nutrition and maternal recognition and its changes can influence early embryonic loss in mares (Bollwein, Mayer, Stolla; 2003).

A high vascular resistance of the uterus in older multiparous mares was shown to occur in result of fibrosis, and can be a cause of infertility in these animals (Bollwein, Maierl, Mayer & Stolla, 1998)

1.5.3 The Uterine Index

The Uterine Index (UI) can be calculated by the difference between the RI values (resistance index) of the uterine arteries from the non-pregnant (RI_{np}) and the pregnant (R_p) uterine horn multiplied by 100.

$$\text{Uterine Index (UI)} = (RI_p - RI_{np}) \times 100$$

In gestation the RI value of uterine artery at the non-pregnant horn is higher from week 4 onward. The UI is positive and becomes higher as pregnancy proceeds. This results from the fact that the uterine horn in which the embryo implants will have a greater blood supply than the horn from the opposite side. The increase in uterine blood flow at the pregnant side can help to develop an optimal endometrial environment and to transport essential nutrients directly to the embryo.

Using the UI, Chen and Stolla (2006) could predict embryonic death as early as 32h before the embryo died, a diagnosis that cannot be currently done by using conventional B-mode ultrasound where measurement of the diminishing embryo size is used. They also established that, if UI value is greater than 10, the pregnancy can be diagnosed as physiologically normal and no embryonic death or interruption of gestation is expected to occur. If the UI value filled between 5 to 10 UI, the same authors recommended a re-examination pre-scheduled time intervals, every 1 to 3 days. If the obtained UI value was lower than 5, it clearly indicated that embryonic mortality was ongoing. This study demonstrates that this method allows the clinician to early detect the condition through and than be able to treat the animal in order to prevent embryonic death (Chen & Stolla, 2006).

1.5.4 Colour Doppler for follicular development and ovulation predicting

In a study about follicle deviation and selection it was shown, by using colour Doppler US, that the peak systolic velocity (PSV) and the time-averaged maximum velocity began to decrease in the future largest subordinate follicle and continued to increase in the future dominant follicle. This difference started 2 days before the beginning of the most evident difference between the subordinate and the dominant follicle (Acosta *et al.*, 2004). These findings allow the veterinarians to identify the dominant follicle before diameter deviation.

Other work, based on a B-mode US, describes an anechoic band progressively, which increases in the area between the theca layers during the 3 days before ovulation (Gastal *et al.*, 2006). During the last 4h before ovulation the same authors showed, with colour-Doppler displays, that a progressive decrease in the percentage of the follicle wall with decrease on colour signals happen. In another study (Carnevale *et al.*, 2002), several mean pixel values

increased in an approximately linear fashion during the 14h before ovulation in hCG –treated mares. These may allow the practitioners to predict with some degree of assurance the time of ovulation and the best time for insemination.

1.6 Power Doppler quantification

Quantification of Power Doppler Energy (PDE) in two-dimensional ultrasound machines has been a subject of interest in recent years and has ranged from subjective methods to semi quantitative techniques (Amso *et al.*, 2001).

Some software are able to capture and make an objective quantification of the PDE image by converting pixel information, in a previous storage image, into linear measurements, allowing true comparisons between regions and permitting calculation of the relative perfusion (Blomley *et al.*, 1997; Yang *et al.*, 1999).

With this software some indices can be calculated and evaluated. Some of these indices include: the region of interest (ROI) area, the area within the ROI occupied with color pixels – colour Doppler area (CDA), the mean colour energy (MCE), the peak colour energy (PCE), and the integrated colour energy per square millimetre. All these parameters represent a true reflection of the vascular density or vascularity within a tissue being analysed at any given point in time (Amso *et al.*, 2001).
Power Doppler ultrasonography for the evaluation of vasculature of CL in women (Miyazaki *et al.*, 1998) and in mares (Bollwein *et al.*, 2002; Hendriks *et al.*, 2006) has been previously described.

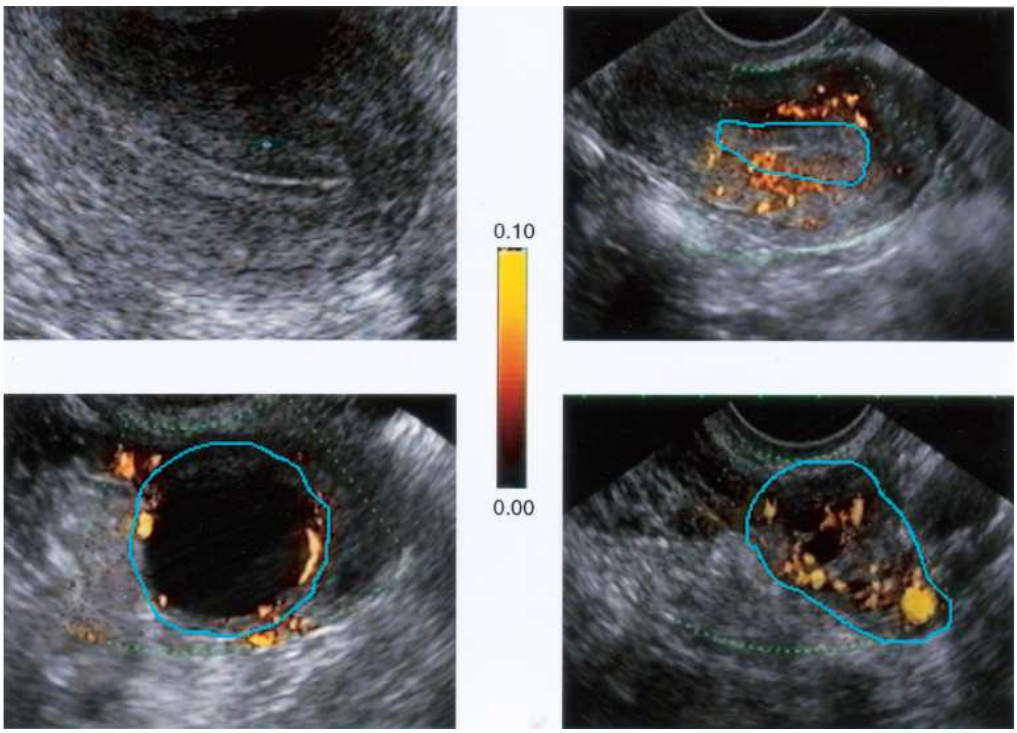


Fig 5 - Ultrasound colour power Doppler images. Top left: transvaginal gray-scale image of endometrium. Top right: ROI drawn around (adapted from Nazar *et al.*, 2001)

Estrous cycle in mares

The mare is a seasonally polyestrous species that normally ovulates during the months of Spring and Summer (Nagy, Guillaume, Daels; 2000). One of the most important aspects on the regulation of its reproductive patterns is the photoperiod (Ginther, 1992). Melatonin secretion increases at the beginning of the dark phase and decreases rapidly at the end of the night (Guillaume and Palmer, 1991; Palmer and Guillaume, 1992). During the dark phase melatonin secretion is stimulated by norepinephrine (Sharp, Grubaugh, Berglund, Seamans, 1980). During Fall and Winter the decrease on day length, which occurs in high latitude located regions, causes an increase in daily duration of melatonin secretion, which results in the decrease of GnRH and cessation of ovulation (Nagy *et al.*, 2000).

It is known that, in addition to photoperiod, factors such as nutrition, body condition and environmental temperature have an effect on seasonal reproductive activity. In fact, more recent studies revealed a complex neuro-endocrine system regulating seasonal changes in hypothalamic and pituitary activity that involves not only melatonin but also neurotransmitters such as opioids and catecholamines (Nagy *et al.*, 2000)

The gonadotropin releasing hormone (GnRH) secretion that occurs on the hypothalamus, results on the releasing of the gonadotrophins as luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Ginther, 1992).

Ovulation is dependent on LH as it is in other species and it reaches its maximum concentration on circulation one or two days prior to ovulation (Ginther, 1992).

Follicle stimulating hormone (FSH) concentrations, which remains low during estrus, increase during diestrus and start to decrease approximately 8 days before ovulation. It is a potent follicular stimulant, which acts by stimulating estradiol production by granulosa cells from the preantral follicle (Ginther, 1992).

Folliculogenesis in mares is characterized by one or two follicular waves (Ginther, 1992). Follicular waves refer to a several follicles that emerge and initially grow in synchrony. In a major wave, the largest follicle of the wave attains the diameter of a dominant follicle (larger than 28mm). In minor waves the largest follicle does not become dominant (Ginther, Beg, Gastal, Gastal; 2004). The follicles that result from major waves may eventually dissociate or deviate. A preferential growth of one, or occasionally two, members of the wave characterize this deviation. This follicle is then termed the dominant follicle (Ginther, 1992). This selected follicle grows to a large diameter (>28mm) and may then either regress (anovolatory major wave) or ovulate (ovulatory wave). The remaining follicles, termed subordinate follicles, undergo atresia (Ginther *et al.*, 2004).

A cascade of intrafollicular biochemical events precedes the beginning of diameter deviation. The mechanism which switches this functional cascade involves reduced circulating FSH concentrations and the attainment of a critical developmental stage by the future dominant

follicle, which includes the acquisition of granulosa cell LH receptors and enhanced responsiveness to gonadotrophins (Ginther *et al.*, 2004). The role of FSH, after the peak of the surge involves the continued growth and development of all follicles before deviation and the developing dominant follicle after deviation (Ginther *et al.*, 2004).

At the time of ovulation, the preovulatory follicle grows up to a maximum diameter, reaching the ovulatory fossa, becoming softer and more sensitive to palpation as ovulation approaches (Ginther, 1992). All ovulations in mares occur through the ovulation fossa. The ovulatory follicle develops, increasing not only in width but also in depth as they extend through the ovarian stroma and encroach on the ovulation fossa (Youngquist, 1997).

1.6.1 Corpus haemorragicum

Immediately after ovulation, the distinct cavity of an ovulation depression can be palpated transrectaly. However it may not be evaluated in some mares even shortly after ovulation. As in most of the cases, blood fills this ovulatory space quickly forming the corpora haemorragica (CH). This structure appears on the ultrasonography as echogenic particle containing which results from blood filling the former follicular space. The CH feels softer and more fluctuant than the surrounding ovarian stroma during the first one to two days after ovulation. As the blood organizes, its ultrasonography appearance becomes more hyperechoic and, over the span of 2 to 4 days more uniform (Youngquist, 1997).

The hypothesis that formation of a CH is necessary in the development of a CL was not supported in some studies (Pierson and Ginther, 1985). It appears that the CH is not functionally important, since it developed in only an half of the luteal glands and its presence did not alter the length of the time that the luteal gland was observed by ultrasonography neither the length of the interovulatory interval (Pierson and Ginther, 1985).

1.6.2 Corpus albicans

As regression begins, the luteal structure undergoes to a lighter appearance because of decreasing vascularisation and increasing connective tissue organization (Ginther, 1992). During the subsequent diestrus the corpus albicans continued to regress, decreasing its size and the pigments residues are condensed. The structure becomes darker with hues of orange, red, or brown. As the involution continues the luteal structure loses its weight and its colour changes from pale yellow to light brown as the cycle progresses (Ginther, 1992).

2.2 Corpus luteum functions

The corpus luteum (CL) is a transient endocrine gland, whose primary secretory product, progesterone (P4) is required for the establishment and maintenance of pregnancy (Webb *et al.*, 2002). The importance of P4 concentration during the first weeks of pregnancy has been demonstrated in cattle (Mann & Lamming, 1999). In agreement with some reports, the presence of an early peak of P4, within 5 days after mating or AI facilitates the elongation of the conceptus and, consequently, the secretion of interferon-tau (Plante *et al.*, 1989), which extends the lifespan of the CL by suppressing estradiol receptor and oxytocin receptor genes (Spencer & Bazer, 1996), and by attenuating the endometrial secretion of PGF2α (Helmer *et al.*, 1989).

Large luteal cells are known to be the source of luteal oxytocin (Sawyer *et al.*, 1986) and relaxin (Fields *et al.*, 1980) in ruminants. Watson and Sertich (1990) demonstrated the production of prostaglandin (PGF2α), PGE_2, and 6-keto-$PGF_1\alpha$, by equine luteal cells. However the role of these compounds in controlling production of progesterone has not been elucidated for the mare. They suggested that the ratio PGF: PGE_2 throughout the cycle may be of significance in luteolysis.

2.3 Luteal insufficiency

The importance of the ovaries and progesterone for the survival of the embryo, including its early stages, has been studied by ovariectomy and by administration of PGF2α to induce luteolysis. This study indicated that progesterone is essential to the survival of the embryo, including the early stages (Ginther, 1985).

Even though, luteal progesterone is essential for various mechanisms associated with early pregnancy, it is not clear whether a deficiency in luteal development or progesterone production can result in a natural reduced pregnancy rate. Results of a study show that, while the progesterone concentration may have contributed to pregnancy or has been a consequence of it, it was not a limiting factor (Sevinga *et al.*, 1999). Contradictory results on primary luteal insufficiency as a cause of failure of pregnancy establishment have been obtained in horses. An effect of defective vascularisation of the corpus luteum on reduced pregnancy rate apparently has not been considered in any species (Silva *et al.*, 2006).

Some authors pointed that failure to maintain a CL and an inadequate progesterone production (≤ 2ng/ml) are the major causes of infertility and early embryonic loss since progesterone is a necessary requirement for both endometrium development and embryo survival (Ginther, 1985; Martin and Laurence, 1994; Webb *et al.*, 2002). As in so many aspects of reproductive physiology, the mare is an exception to the majority rule. Its primary

corpus luteum of pregnancy begins to decline in secretory activity as early as day 14 –16 after ovulation and thus at about the same time that complete luteolysis would normally occur in the cycling animal. This causes a slow fall in peripheral plasma progesterone concentrations during the next 20-25 days until a secondary rise commences, usually at around day 35-45 coincidentally with the onset of secretion of equine Chorionic Gonadotrophin (eCG) (Allen, 1984). This second rise on progesterone concentration attributed to the development of secondary corpora lutea that develops in mare's ovaries between days 40 to 150, and to the horse placenta that secretes sufficient of progesterone to maintain pregnancy from about day 100 (Allen, 1984).

2.4 Corpus luteum formation

In most species the CL develops from the granulosa and theca interna and externa cells after the ovulation. However, in the mare, the cells from the theca interna do not contribute to luteal tissue as seen in other farm species (Ginther, 1992).

2.5 Corpus luteum morphology

In the equine corpus luteum the morphology is unlike that of ruminants, in that there is marked trabeculation of tissue that results from the collapse of the preovulatory follicle at ovulation (Lawler, Hopkins, Watson; 1999). These trabeculae consist of extracellular matrix, fibroblasts and small cells (Harrison, 1946), and contain much of the vasculature of the corpus luteum. The steroidogenic large cells of the equine corpus luteum are thought to have origin only from the granulosa layer of the follicle (Van Niekerk, Morgenthal, Gerneke, 1975)

After ovulation, in cows, granulosa cells differentiate into large luteal cells (LLC), which comprise 30% of steroidogenic cells, and secrete 70% of progesterone, even though LH does not stimulate them for the progesterone secretion. In ruminants, the theca interna differentiates into small luteal cells (SLC), which includes 70% of steroidogenic cells. Small luteal cells require LH stimulation for maximal progesterone secretion, but only secrete 30% of all progesterone (Farin *et al.*, 1989; Niswender *et al.*, 1994). In the mare CL it has been shown that it is composed mainly of three types of cells: large luteal cells, small luteal cells, and endothelial cells. It has been shown, in the same species, that the proportion of LLC decreased between mid and late diestrus, and the proportion of SLC increased as age of CL advanced (Watson and Sertich, 1990). Nevertheless, contradictory results indicated no changes in SLC number with CL development and lysis, while LLC increased from early to mid luteal phase (Roberto da Costa *et al.*, 2005)

In contrast with other species, in the mare, small luteal cells are not of thecal origin (Van Niekerk *et al.*, 1975; Ginther, 1992), since thecal cells undergo regression within 24h after ovulation (Van Niekerk *et al.*, 1975).

The repeated mitoses of steroidogenic cells, in the developing CL, are accompanied by a highly intense angiogenic process, which reaches a peak 2-3 days after ovulation (Reynolds, Grazul-Bilska, Redmer; 2000). Thus, the majority of steroidogenic cells of the mature CL are in contact with one or more capillaries (Reynolds, Killilea, Redmer, 1992).

In another study on equine CL, there was an increase in proliferating cell nuclear antigen expression (PCNA) in large luteal cells from the corpus haemorrhagicum to the mid luteal phase, followed by a decrease towards the late luteal stage (Roberto da Costa *et al.*, 2005).

The normal development of the CL and its capacity to produce progesterone, growth factors and angiogenic factors depend on its vascularisation (Acosta & Miyamoto, 2004). This neovascularisation is important to provide the circulating substrate, such as low-density lipoprotein, that is used by the luteal cells for progesterone biosynthesis (Carr *et al.*, 1982).

The sprout of new blood capillaries supports the development of luteal cells and appears to be locally potentiated by Angiotensin II (Ang II) and growth factors that induce angiogenesis and support the synthesis of progesterone in luteal cells (Kobayashi *et al.*, 2001).

2.6 Local control mechanisms

2.6.1 Luteal angiogenesis

The rapid cyclical changes in luteal growth and regression demands corresponding rapid changes within its vasculature. It is well known that regulation of angiogenesis is a critical factor regulating luteal function (Webb *et al.*, 2002).

Angiogenesis consists of at least three steps; break-down of the basement membrane of existing blood vessels; migration of endothelial cells towards an angiogenic stimulus and proliferation of endothelial cells to establish a new blood vessel sprout (Redmer *et al.*, 2001).

Studies in ruminant's CL showed that the main angiogenic factors are the basic fibroblast growth factor (FGFs) and vascular endothelial growth factor (VEGF). These factors stimulate Ang-II, PGF2α, and progesterone secretion (Reynolds *et al.*, 1999)

2.6.1.2 Vascular Endothelial Growth Factor

There are some evidences that VEGF is the main angiogenic factor in the corpus luteum (Reynolds and Redmer, 1998). In the mare, luteal cells express VEGF until the late luteal phase, when its expression decreases (Tamanini and Ambrogi, 2004). VEGF has been

demonstrated for the first time in neutrophils in the equine CL, by immunohistochemistry, although its mRNA expression has not been detected (Al-zi'abi, Watson, Fraser; 2003).

Although VEGF may be induced by hypoxia in most ischemic tissues, in the corpus luteum, it is probably mostly regulated by LH (Neulen *et al.*, 1998; Dickson and Fraser, 2000). In the human CL, LH and human chorionic gonadotrophin (hCG) influence the production and different actions of the VEGF. In women, hCG is implicated on the maintenance of the CL during pregnancy (Wullf *et al.*, 2001) and LH reduction decreases VEGF levels.

In a study on the equine CL (Al-zi'abi *et al.*, 2003), it was shown that a marked endothelial cell proliferation occurs during the early and mid-luteal phases, and the high expression of VEGF mRNA and protein occurs at these periods. This fact, associated with a decrease in both VEGF and endothelial cell proliferation during natural luteolysis is strongly indicative of a paracrine role for VEGF in the regulation of blood vessel growth and development in the corpora lutea of mares (Al-zi'abi *et al.*, 2003).

In the mare, the high proliferation of endothelial cells in the early CL leads to increased vascularization as it becomes more mature. The dense vasculature that is present in the mid-luteal phase is required for the release of progesterone precursors to, and progesterone from, the luteal cells (Al-zi'abi, Fraser, Watson; 2002). PGF2α administration seems to reduce VEFG expression by inhibiting endothelial cell proliferation (AL-ziabi *et al.*, 2003).

VEGF may play a role in non-angiogenic functions in corpus luteum as well, such as regulation of vascular permeability, vasodilatation, and mediation of endothelial cell survival after PGF2α administration (Folkman & Klagsburry, 1991, Goede *et al.*, 1998; Berisha *et al.*, 2000). It also may play an indirect role in neutrophil migration into the regressing corpus luteum by stimulating the release of chemokines (Lee *et al.*, 2002). As VEFG stimulates increased vascular permeability, angiogenesis and endothelial cell mitosis (Folkman and Klagsburn, 1991), it is evident that it plays a very important role in regulating angiogenesis and luteolysis in the CL (Al-zi'abi *et al.*, 2003).

2.6.1.3 Basic Fibroblast Growth Factor bFGF

Basic fibroblast growth factor (bFGF) was the first angiogenic factor identified in the ovary (Gospodarowicz, Cheng, Lui, 1985). The bFGF is expressed in both mature follicles and CL with little variations during the ovarian cycle. It stimulates endothelial cell proliferation (Bikfalvi *et al.*, 1998) and it exerts an anti-apoptotic effect on granulosa cells, favouring the production of angiogenic factors and inhibitting nitric oxide (NO) production (Grasseli *et al.*,

2002). In the luteal phase, bFGF does not play a critical role in physiological angiogenesis since its absence is compensated by other factors (Tamanini & Ambrogi, 2004). bFGF also seems to be involved in the induction of luteolysis as it stimulates prostaglandin secretion in bovine luteal cells (Neuvians *et al.*, 2004).

2.6.1.4 The IGF system

Insulin-like growth factors (IGF-I and II) are widespread homologous polypeptide growth factors that play important roles in growth and development. IGF is also regulated by specific interactions with IGF binding proteins, regulating their half-life and clearance (Armstrong & Webb, 1997; Webb *et al.*, 2002).

The IGF's are important promoters of steroidogenesis with the potential to act at multiple sites. IGF-I and II may also regulate luteal angiogenesis and apoptosis. The interaction of the IGF receptor with IGF-I or – II has been shown to protect a range of cell types, including ovarian cells, from apoptosis. They modulate luteal cell function via interaction with specific cell surface receptors (Webb *et al.*, 2002).

2.6.1.5 Other angiogenic factors in the luteal phase

During the luteal phase, nitric oxide (NO) produced by endothelial cells increases blood flow by stimulating arteriolar smooth muscle relaxation and as such favours angiogenesis through an increase in VEGF production by capillary pericytes (Tamanini *et al.*, 2003). However, NO may also play a luteolytic effect inhibiting progesterone and stimulating prostaglandins secretion (Neuvians *et al.*, 2004). It has been shown that VEGF, FGF, endothelial growth factor and epidermal growth factor are important modulators of NO synthesis (Roberto da Costa *et al.*, 2007). In the mare, NO seems to be involved in follicular growth and ovulation (Pinto *et al.*, 2002). Furthermore, it has been involved in the autocrine/paracrine luteolytic cascade induced by PGF2α in cows (Skarzynski *et al.*, 2003). Therefore, it appears that inhibition of angiogenic activity in the equine CL by exposure to long-term progestagens might be also a NO mediated process (Ferreira-Dias *et al.*, 2006), since the the decrease of BAEC proliferation in the mid-luteal phase CL, incubated in presence of P4 and pregnenolone, was accompanied by a lower production of NO.

Angiotensin II (Ang II) is a vasoactive peptide, converted from Ang I by angiotensin-converting enzyme (ACE), that regulates oocyte maturation, ovulation and steroidogenesis. In bovine CL, endothelial cells possess Ang II receptors and can convert Ang I to Ang II (Kobayashi *et al.*, 2002). This conversion takes place on the surface of endothelial cells (Kobayashi *et al.*, 2002).

Endothelin 1 (ET-1), which concentration levels are higher after ovulation and after CL regression, associated with Ang II may act as a vasoconstrictor and apoptosis inducer during luteolysis (Schams *et al.*, 2003).

2.7 Anti-angiogenic factors in luteal phase

In the mare, in the mid luteal phase CL, when luteal growth is complete, there is a decrease in endothelial cell proliferation, in the presence of progesterone or its precursor (Ferreira-Dias *et al.*, 2006). This could be due not only to lack of synthesis of angiogenic factors, but also to a rise in anti-angiogenic factors, such as thrombospondins, angiostatin and endostatin (Hazzard *et al.*; 2002).

Thrombospondins are extracellular glycoproteins that have functions on platelet aggregation, inflammatory response and the regulation of angiogenesis during wound repair and tumour growth (Adams and Lawler, 2004). These glycoproteins may play an anti-angiogenic effect by inhibiting endothelial cell proliferation in the CL (Bagavandoss and Wilks; 1990).

Angiostatin is an internal proteolytic fragment of plasminogen that was first purified from a mice bearing a Lewis lung carcinoma (O'Reilly *et al.,* 1994a). Angiostatin inhibits tumour angiogenesis and induces tumour dormancy, inhibiting endothelial cell proliferation (O'Reilly *et al.*, 1994b). The action of angiostatin appears to be endothelial specific (Chen *et al.*, 2003). In addition to its anti-proliferative effect, angiostatin also induces cells to undergo apoptosis (Cao *et al.*, 1996). It appears that angiostatin also play its antiangiogenic role diminishing the activation of bFGF or VEGF in the endothelial cells, but not in other cell types (Redlitz, Daum, Sage; 1999). Angiostatin also activates the Fas-mediated apoptotic pathway in part through the up-regulation of FasL mRNA, down-regulation of c-Flip and activation of caspase-3 (Chen *et al*., 2003). Caspases regulate the selective destruction of structural and functional key proteins in the cell (Thornberry *&* Lazebnik, 1998). Caspases have been shown to be involved in luteal regression in the cow (Rueda *et al.*, 1997), sheep, (Rueda *et al.*, 1999) and other animals. In mares, caspase-3 might play an important role during luteal tissue involution (Ferreira-Dias *et al.*, 2007)

Endostatin is another potent inhibitor of angiogenesis and tumour growth (Reis *et al.*, 2005). In a study it was shown that endostatin decreases proliferation of endothelial cells, inhibits migration of several endothelial cell lines, induces apoptosis, interferes with the interaction between endothelial cells and extracellular matrix metalloproteinases (Reis *et al.*, 2005). These factors may play an important role by modulating angiogenesis during the luteal regression (Maisonpierre *et al.*, 1997; Espinosa Cervantes & Rosado Garcia; 2002).

2.8 Progesterone synthesis

Progesterone is the main secretory product of the corpus luteum. Following LH stimulation, theca interna synthesizes androgens, which subsequently diffuse through the basement membrane. Within granulosa cells, androgens are converted to estradiol-17β by the aromatase enzyme, which is under the control of follicle –stimulating hormone (FSH) (Squires, 1993).

The binding of LH to its receptor promotes progesterone secretion, by activating adenyl-cyclase, which synthesizes cAMP, an intracellular messenger that is responsible for activating the enzymes involved in progesterone synthesis (Niswender *et al.*, 1994; Weems *et al.*, 2006). However in a study, addition of LH, dbcAMP, or ionophore to cell cultures did not consistently affect the secretion of progesterone by the mare's luteal cells (Watson and Sertich, 1990)

The cholesterol necessary for progesterone synthesis may be obtained from high or low-density lipoproteins. An unesterified form associated with the membranes is also present in the luteal cells. An esterified and stored form is present in the small and large luteal cells in lipid droplet form (Hansel, Alila, Dowd, Yang, 1997).

In mares, synthesis of P4 starts early in the luteal structure. This synthesis is also accompanied with the increase in P4 receptors (PR) in large luteal cells, luteal microvascularization, proliferating cell nuclear antigen (PCNA) expression and large luteal cell count (Roberto da Costa *et al.*, 2005). It had been described that P4 has a luteotrophic effect, maintaining the synthesis of this steroid in the ovary. Furthermore it may also play an anti apoptotic effect acting as an autocrine factor, by a PR-dependent mechanism. The long-term effects of exposition to P4 or its precursor, pregnenolone, may inhibit angiogenic factors or stimulate anti-angiogenic factors production by equine mid CL, preparing the equine CL for functional and structural regression. It is possible that inhibition of the angiogenic activity in the equine CL might be NO mediated (Ferreira-Dias *et al.*, 2006).

Furthermore P4 may also affect luteotrophic PGE2 production in the equine CL, controlling the conversion of luteotropic PGE_2 to luteolytic $PGF_{2\alpha}$. These facts may show that P4 is either a luteotrophic and luteolytic factor in the mare (Ferreira-Dias *et al.*, 2007).

2.9 LH and CL

The LH is a pituitary hormone that rises before the ovulation by environmental influence, such as length of photoperiod, since it depends on the GnRH depletion (Irvine & Alexander, 1993), and regulates the CL in a number of species (Ginther 1992).

In the mare, the maximum concentration of this hormone increases until day 2 after ovulation. LH concentration remains low during mid-diestrus, when it begins to rise, a few

days before the onset of estrus, reaching its maximum value after ovulation and then start to decrease until low diestrous values (Ginther, 1992). Unique to the mare, LH remains quite high for several days after ovulation. It is thought that elevated concentrations of LH after ovulation may be important for the development of the CL (Noden *et al.*, 1975)

The rise of the LH levels, in the presence of a preovulatory follicle, stimulates its growth, ovulation and formation of the CL.

The majority of receptors for LH have been reported to be present on small luteal cells (Alexander & Irvine, 1993)

The LH release from the pituitary is regulated by progesterone and estradiol. They inhibit LH secretion by exerting a negative feedback on the hypothalamus. During luteolysis, progesterone levels decrease and the negative feedback influence is lost, promoting the slow rise of LH.

2.10 Luteolysis

It is known that the decrease of ovarian blood flow is one of the PGF2α luteolytic actions (Acosta *et al.*, 2004). After the administration of PGF2-α an acute increase in luteal blood flow happens between 30 min and 2 h in cows (Acosta *et al.*, 2002), suggesting that an initial increase in intraluteal blood flow may trigger the initiation of the luteolytic cascade in the cow (Acosta *et al.*, 2004). As in other females, in mares, luteolysis depends on the pulsatile release of prostaglandin F2α from the endometrium (Ginther, 1992).

Nitric oxide is another local vasodilatory substance that may play a direct luteolytic role in the regressing CL. It has been demonstrated that prostaglandins (PGs) modulates luteal NO synthase (NOS) activity and progesterone production, depending on the stage of the CL (Acosta *et al.*, 2004).

During luteolysis some vascular changes occur including an initial acute increase in the blood flow that is both luteal stage and prostaglandin (PG) dependent and is necessary for the induction of the local release of endotheline 1 (ET-1) (Ohtani *et al.*, 1998; Levy *et al.*, 2000).

In general, cells die by three recognised mechanisms: apoptosis, necrosis or terminal differentiation (Al-zi'abi, 2002). During necrosis the permeability of cells is increased, which causes cellular swelling, non-selective DNA degradation and inflammation in the surrounding tissues (Al-zi'abi, 2002).

In mares, the corpus luteum starts regressing functionally from day 10 to 12 onwards, and luteal cells decrease in size from day 16. By day 20, two types of cells degeneration were reported by histological examination: pyknosis associated with shrunken nuclei and very condensed cytoplasm and karyolysis characterized by lysis of chromatin (Van Niekerk *et al.*, 1975). The corpus luteum shows changes in mass, shape and colour throughout the oestrous cycle (Al-zi'abi, 2002).

In the mid-luteal phase, luteal cells are polyhedral to elongated, and have an abundant cytoplasm and a spherical nucleolus. From day 14, luteal cells start to show degenerative changes and pyknotic cells with dense basophilic nuclei are present between apparently healthy luteal cells. At day 17, all luteal cells decrease in size and contain vacuoles. Fibroblasts and connective tissue infiltrate the corpora lutea, intercellular debris and leucocytes are present (Al-zi'abi, 2002).

Detection of pycnotik luteal cells and round dense bodies, which stain positively with the TUNEL technique, indicates apoptosis during natural and PGF2α-induced regression (Al-zi'abi, 2002). However, crenation of nuclear membrane and shrinkage of nuclei in the luteal cells may indicate an additional form of cell death, which is possibly terminal differentiation. During terminal differentiation, a progressive condensation of nuclear material results eventually in pyknosis and nuclear destruction or expulsion (van Wezel *et al.*, 1999).

Degeneration in mitochondria and SER has been proposed to result in a decline in progesterone production and play a role in the accumulation of lipids. Degenerative changes in the equine CL may be initiated by day 10 starting with mitochondrial rarefaction. Apoptotic bodies and cells are present by day 14 of the oestrous cycle and 12h after PGF2α administration (Al-zi'abi *et al.*, 2002).

Based on a study of Al-zi'abi *et al.* (2002), the rarefied mitochondria, which is indicative of minor degenerative changes observed in some luteal cells during the mid-luteal phase (day 10), is related with the onset of declining concentrations of progesterone (Van Niekerk *et al.*, 1975). So it has been proposed that the degeneration of the mitochondria in the mid-luteal phase in mares is an early step in the decline of progesterone concentrations, since mitochondria are involved in steroidogenesis (Levine, Wight, Squires, 1979). In other studies, it was shown that mitochondrial disruption may reflect an early sign of apoptosis (Kroemer, Dallaporta, Reshe-Rigon 1998).

In contrast with other species (Azmi and O'Shea, 1984), in mares and cows, endothelial cells in the CL do not show any morphological signs of apoptosis. Usually they show swelling and detachment from the walls of the blood vessels, showing that there are species differences in the fate of endothelial cells during luteolysis and, that the vasculature of the CL regresses in a similar manner in horses and cattle (Al-zi'abi *et al.*, 2002).

A marked increase in lipid droplets occurs during luteal regression. It is characteristic of the late regression of the CL, occurring after the onset of structural regression (Al-zi'abi *et al.*, 2002). Accumulation of lipids is associated with CL regression in other species, as in the sheep (Deane *et al.*, 1966), cow (Priedkalns and Weber, 1968), sow (Waterman, 1980) and rat (Guraya, 1975).

2.11 Immune cells in the corpus luteum

Immune cells are a significant proportion of the non-luteal cells present in the CL. These cells, as well as being primarily phagocytic, are involved in regulating luteal function through the integration of immunological and endocrinological mechanisms (Webb, 2002).

In 1968, Lobel and Levy described the presence of white blood cells in the bovine corpus luteum (Pate and Keyes, 2001). They observed the presence of lymphocytes in the connective tissue that surrounded the luteal vasculature on day 14 and, by day 15-17 of estrous cycle, lymphocytes were infiltrated among luteal cells. They also reported that macrophages were already present on day 19 (Pate and Keyes, 2001). Macrophages were later shown to be involved in phagocytosis of cells and cell remnants (Paavola, 1979).

Other studies confirmed the presence of lymphocytes and macrophages in the corpus luteum throughout the luteal phase (Kisch *et al.*, 1981; Hume *et al.*, 1984; Bagavandoss *et al.*,1988).

The recruitment of macrophages into the corpus luteum of rats, rabbits and cows is probably regulated by the expression of monocyte chemoattractant protein 1 (MCP-1) (Bowen *et al.*, 1996; Penny *et al.*, 1998; Penny, 2000). MCPs are produced in the corpus luteum by endothelial cells, fibroblasts and T-Lymphocytes, and show a very potent chemotactic effect for monocytes, that can regulate the population of macrophages during the structural regression of the CL (Penny, 2000). In another study it was shown that intercellular adhesion molecule 1 (ICAM-1) might play an important role in monocytes and macrophages recruitment during luteal regression in rats (Olson and Townson, 2000). So, there are at least two molecules that may be responsible for attraction, activation and promoting adhesion of immune cells in the CL tissue and these are increased at the time of luteal regression in other species (Pate and Keyes, 2001).

The presence and increase of lymphocytes at the time of luteolysis, implies that they may also function in a manner that facilitates regression. Therefore, it is possible that immune cells could be involved in the onset and progression of luteolysis and play a role beyond that of merely cleaning up cellular debris (Bagavandoss *et al.*, 1988). Two hypotheses are proposed. Firstly the immune cells have a direct participation in loss of steroidogenesis and in the demise of luteal cells and tissue. Endothelial cells, fibroblasts and immune cells secrete a variety of potential paracrine regulators of ovary function, such as growth factors, prostaglandins and lipoxygenases. Activated macrophages and T-lymphocytes, secrete cytokines, which potentiate the immune response. These cytokines may exert a positive or a negative feedback on endocrine cell function (Wuttke *et al.*,1997).

Apoptosis is apparently inhibited by progesterone in the bovine CL (Rueda *et al.*, 2000), and is initiated by TNF-α in some type of cells, including luteal cells. As TNF-α and its receptors are present in the corpus luteum, it is proposed that cytokines may promote apoptosis of

steroidogenic cells directly mediating the luteolytic action of PGF2α (Pate and Keyes, 2001), and as such, TNF-α may promote luteal cell apoptosis (Friedman *et al.*, 2000). Some data support a role for TNF-α in facilitating luteolysis after PGF2α causes an initial decline in progesterone (Pate and Keyes, 2001).

Reactive oxygen species (ROS), which production is stimulated by PGF2α and NO, have been implicated as mediators of cell death during luteolysis (Pate and Keyes, 2001).

Immune cells may play a primary role in CL by activating Fas, a transmembrane receptor that belongs to the TNF receptors family, and initiate cell death (Pate and Keyes, 2001).

Luteal cells are able to express class I and class II major histocompatibility complex (MHC) molecules (Fairchild and Pate, 1989; Pate and Keyes, 2001), and when are exposed to TNF-α or IFN, the expression of class I MHC per cell is enhanced (Benyo and Pate, 1992). As there are evidences that MHC are functional immunologically molecules, it is suggested that luteal cells could serve as antigen-presenting cells, initiating a transient autoimmune response during luteolysis (Petroff *et al.*, 1997).

The second hypothesis defends that immune cells protect surviving luteal cells as the corpus luteum regresses. It is proposed that these cells control a potential inflammatory condition created by dead and dying cells, to provide a beneficial environment for the cells that have not died, and participate in a continuous restructuring as the corpus luteum loses mass (Pate and Keyes, 2001) So, it is assumed that the CL regresses primarily through the loss of cells by apoptosis (Rueda *et al.*, 1997), and it is followed by phagocytosis by macrophages (Savill *et al.*, 1993).

It was shown that the immune cell population in the equine corpus luteum varies during the estrous cycle and after administration of PGF2α (Lawler, Hopkins, Watson, 1999). It was thought that macrophages and other leucocytes were only involved in removing luteal cells by phagocytosis during structural regression. However, macrophages secrete a wide variety of products that are involved in connective tissue breakdown and vascular changes and are ovulatory mediators (Brannstrom *et al.*, 1994). Macrophages are a very important source of multifunctional cytokines such as TNFα and IL-1, which can influence ovarian cell function (Lawler *et al.*, 1999). It was shown that influxes of macrophages precede the functional luteolysis, which may show a possible role for macrophages and secretory products in functional and structural luteolysis (Lawler *et al.*, 1999).

CD8+ cytotoxic T cells are increased just before functional luteolysis (days 12-14), which may indicate a role of these cells and their products in luteolysis, unlike CD4+, that is increased in the early and mid-luteal phase, which may suggest that they do not play an important role on luteolysis. In the same study it was shown that eosinophils were present in the equine corpus luteum in a very small amount throughout the estrous cycle, and showed no significant increase at luteolysis (Lawler *et al.*,1999).

Corpus luteum evaluation and imaging

Due to the anatomical configuration of the mare's ovary, transrectal palpation of the CL *per rectum* is not feasible. In fact, in the mare the CL forms inside the ovary and does not protrude above the surface of the ovary (Squires *et al.*, 1988).
In fact, by using a real-time B-mode US, the mare's CL can be identified for an average of 17 days after ovulation or until five days before the subsequent ovulation (Pierson and Ginther, 1985). The ultrasonic properties of some mature corpora lutea are similar to those of ovarian stroma. However, luteal tissue can be normally, distinguished from the stroma by a margin which is formed in part by the difference in acoustic impedance of the tissues (Pierson and Ginther, 1985).

Approximately 50% of CL are characterized by a nonechogenic central cavity, filled with blood (Pierson and Ginther,1985). Despite the morphologic differences between the luteal structures, with and without centrally echogenic area, this does not appear to be functionally significant, since the amount of luteal tissue is not different between the two types of CL and it does not interfere with the length of interovulatory intervals (Townson, Pierson, Ginther, 1989).

The gray-scale brightness and contrast, according with the echogenic portion of the luteal gland, appears to give an indication of luteal hemodynamics (Pierson and Ginther, 1985). The ultrasound images of the CL are affected by the amount of blood contained within it. Blood is semi-echogenic and serum is non-echogenic, whereas the opposite luteal stroma is echogenic. Generally, luteinisation is observed first on the periphery of the luteal structure. As the CL ages and blood is normally reabsorbed, a solid luteal structure that is uniformly echogenic develops. In general the CL is mostly echogenic on day 0 or 1 post-ovulation. This probably results from the ultrasonic properties of the collapsed follicle (Squires *et al.*, 1988).

The echogenicity of the CL decreases over the first six days of diestrus, remains at a minimum level for several days during the middle of diestrus and then increases over days 12 to 16 (Pierson and Ginther, 1985). This increase of brightness during CL regression is indicative of a decreased blood flow, increased tissue density, and fibrin infiltration (Pierson and Ginther, 1985).

Some of the indications for ultrasound evaluation of the CL are: (1) to detect ovulation; (2) to evaluate CL formation; (3) to determine size and characteristics of the CL; (4) to determine if the failure of a mare to display estrus is caused by the prolonged maintenance of a CL or by the absence of a CL and follicular activity; (5) to distinguish between anovulated

haemorrhagic follicles, luteinized unruptured follicles, and CL; and finally (6) to determine if a mare has ovulated more than one follicle.

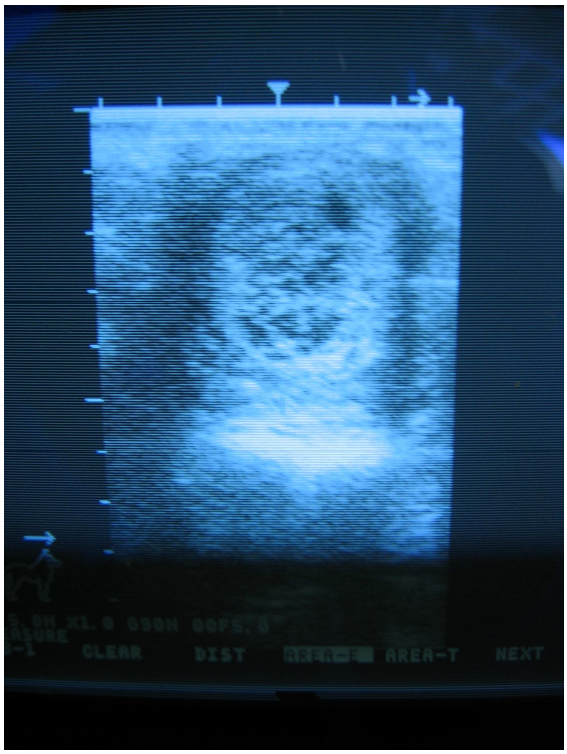


Fig 6 - Ultrasound of a CH few hours after ovulation;

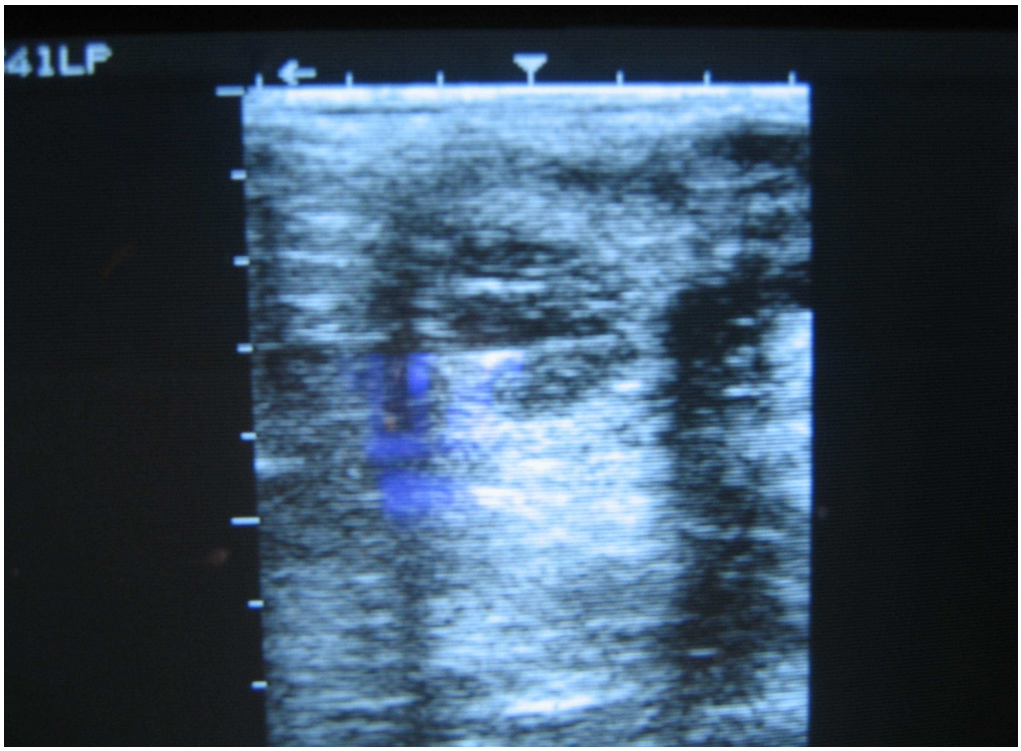


Fig 7 -Corpus luteum in gray-scale ultrasound (Aloka500)

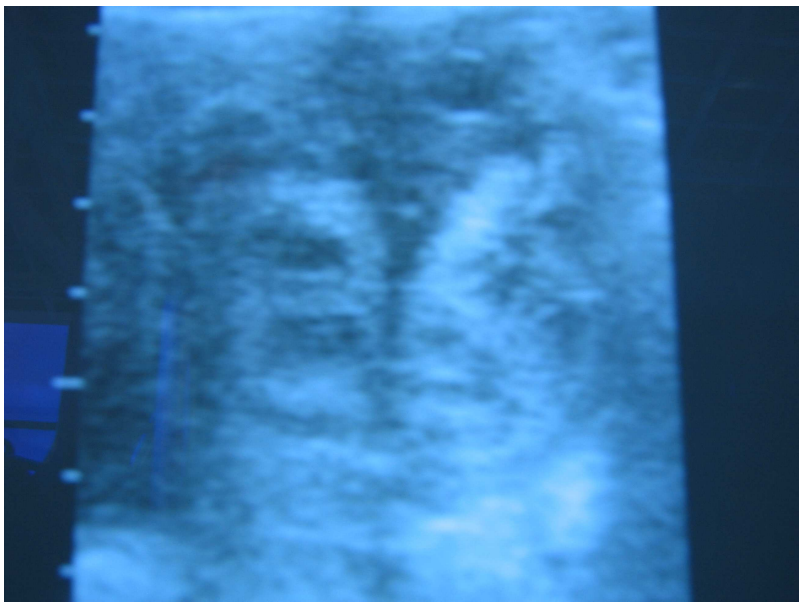


Fig 8 -- Formation of two CL after double ovulation;

The CL area can be estimated by measuring its height (H) and width (W) and applying the equation $A = 0{,}5H \times 0{,}5W \times \pi$ (Bollwein *et al.*, 2002).

Evaluation of the size of the luteal tissue may be important, because its size can affect gland's productivity (Townson and Ginther, 1989). Pierson and Ginther (1985), suggested that the intensity of luteal echogenicity could reflect the extent of luteal hemodynamics and therefore productivity. Ginther (1992) also observed that the period of diminished progesterone production at the beginning of the end of diestrus, coincides temporally with higher luteal-echogenicity scores, whereas high progesterone production during mid-diestrus coincides with lower echogenicity scores.

Some studies have already tried to classify the luteal gland tissue with the gray scale by subjective scoring and by pixel analysis (Townson and Ginther, 1989). Townson and Ginther (1989) described, with the gray-scale assessment, that the first increase in gray-scale score occurred between 5h and 24h after the onset of luteal development and reached its maximum at 48h. The same authors did not find significant differences between the results of pixel analysis in assessing the intensity of luteal echogenicity and the visual results of gray-scale scoring, a more subjective method.

Relationship between gray scale characteristics and progesterone production had already been studied before (Bergfelt & Ginther, 1996). Bergfelt & Ginther (1996) described an inverse temporal relationship between echogenicity of the corpus luteum and circulating concentrations of progesterone in horses and ponies. However, Arruda *et al.* (2001) found a parallel relationship between the morphoechogenicity and plasma progesterone concentrations in embryo recipient mares.

1.7 Assessing corpus luteum vascularisation

In the past, changes in the CL during the estrous cycle in the mare could be monitored only by using B-Mode sonography (Ginther, 1992) and by determining plasma progesterone levels (Townson *et al.*, 1989).

In recent years colour Doppler sonography, including using power Doppler mode has been used in human obstetrics to examine, noninvasively blood circulation of the CL. This technique can detect physiologic changes in blood flow of the ovary in the luteal phase and is now considered a useful non-invasive tool for evaluating CL function (Miyazaki *et al.*, 1998) and infertility problems in women (Borini *et al.*, 2004).

In other species, a positive correlation between cyclic changes of luteal blood flow and plasma progesterone levels has also been found. However, this was only showed by the use of invasive techniques such as implanting electromagnetic flow probes or by the injection of radioactive microspheres (Bollwein *et al.*, 2002).

Bollwein *et al.* (2002), by using power Doppler to evaluate luteal blood flow changes in mares during the estrous cycle, reported that CL circulation reached its maximum on day 5 post-ovulation, before the rise of progesterone concentration in the peripheral blood (day 7 post-ovulation). This difference in days between both occurrences may suggest that a strong luteal vascularisation is an important prerequisite for the growth and differentiation of luteal cells, and the development of hormone receptors (Bollwein *et al.*, 2002). This technique can also be helpful in the evaluation of inadequate luteal vascularisation as a cause of infertility disorders in mares.

Materials and methods

Animals

Nine cycling French Trotter mares (n=9) were used in this trial from January to April, during the transition season from Invernal anestrus. Mares were not examined sequentially, and most of them were evaluated only one time, with seventeen exams perfomed throughout the study. When more than an evaluation was performed in an individual mare they were identified as **a, b, c**… The ovulation time was unknown. All mares were housed in boxes and kept under artificial light from 8:00h until 18:00h. They were fed with hay and supplemented with concentrates. Water was provided *ad libitum*.

Ultrasonography

The ultrasound evaluation was performed using an ultrasound (MyLab 30 PieMedical®) with a linear array transducer (7.5MHz, pulse Rep freq 3KHz, colour gain 70%). For these purposes the mares were conducted to the evaluation room, where the restraining stocks were placed. After the removal of faeces from the rectum, transrectal examination was preformed to evaluate the size and tone of the uterus and to locate the position of both ovaries by palpation (Squires *et al.*, 1988). The ultrasound probe was then placed in the rectum and moved across the reproductive tract in the following pattern: bladder, uterine body, right uterine horn, right ovary, right uterine horn, uterine body, left uterine horn, left ovary.

Whenever luteal tissue could be visualized by ultrasound, the following measurements were made: cross diameters (D1, D2), area and volume of the luteal tissue, Doppler colour flow and Power Doppler measurement of the luteal tissue.
Power Doppler images were stored at the optimal recording, with the maximum area, without flash artifacts, and with the maximum number of colour pixels in the CL.

Progesterone assay

Blood samples were obtained from the jugular vein within minutes after ultrasound exam (Vacutainer Systems; Becton Dickinson & Co, Franklin Lakes, NY, USA) and were centrifuged at 2000 x g. Plasma was stored at -18℃ to be analysed for progesterone (P4) by radioimunoassay (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA).

Statistical analysis

Plasma progesterone concentrations, CL diameters, cross-area and volume of CL were statistically analyzed by SAS PRO CORR (Version 9.1;SAS Institute Inc., Cary, NC, USA). Those measurements were compared using the correlation coefficient (Pearson's correlation) and assuming $P<0.05$ as the level of significance.

Fig 9 and 10- Stables and examination room at Ghent faculty;

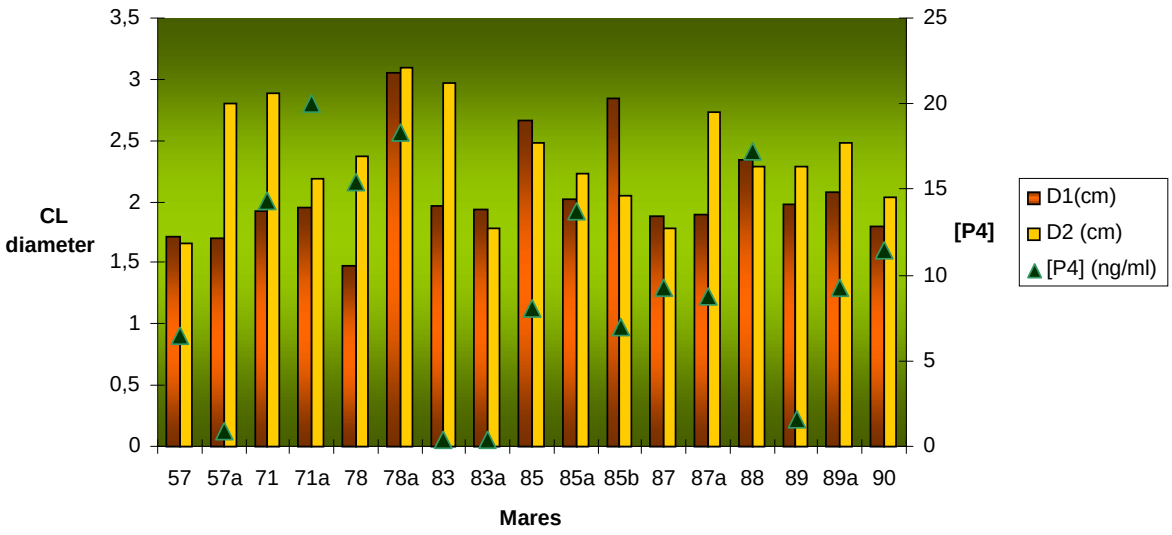
Results

In this study, three measurements of progesterone (P4) concentration in the plasma were < 1.0 ng/mL (0.86 ng/mL; 0.42 ng/mL; 0.40 ng/mL). The concentrations of plasma progesterone ranged from 0.40 ng/mL in mare 83, in measure **a**, and 20.0 ng/mL in mare 71, in measure **a**.

The cross diameters (D1, D2) of CL were assessed by two different veterinarians, and a mean value was estimated. A minimum value of 1.41 cm was observed in mare 78, and a maximum of 3.1 cm in the same mare (**78a**) 3 days after the first evaluation.

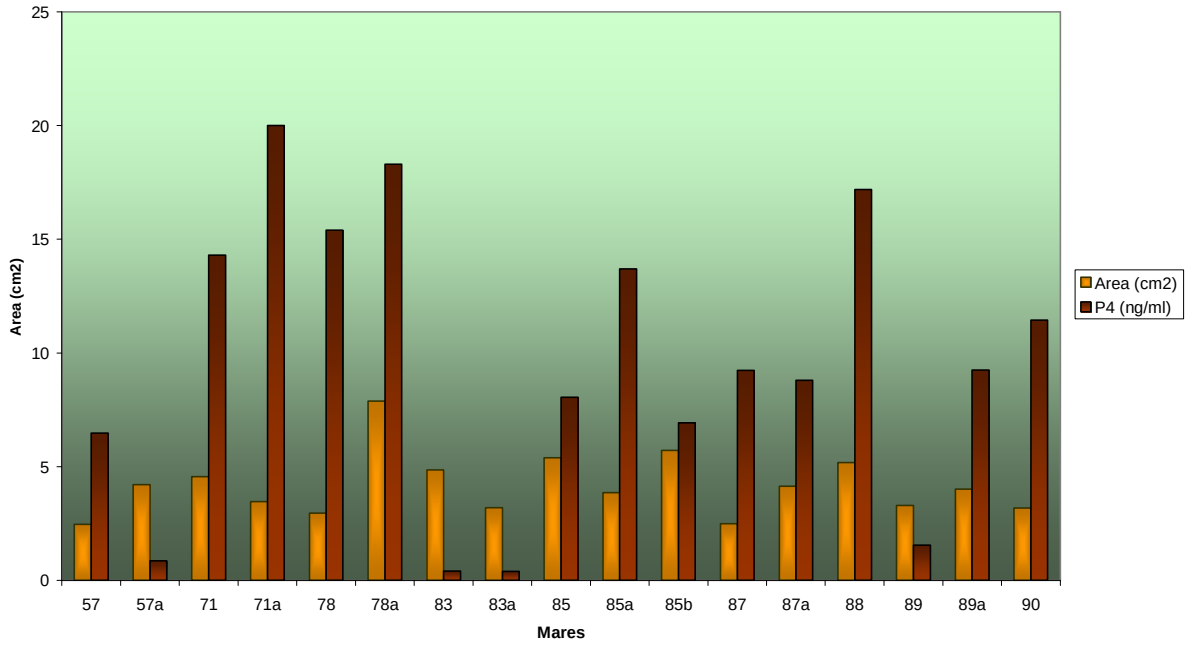
The areas of the CL were also assessed and the values were comprised between 2,47 cm^2 (**57**), and 7,88 (**78a**).

The values for the volume of the luteal gland ranged from 2.8 ml, in mare 57, and 15.5 mL in mare 78, in measurement a.



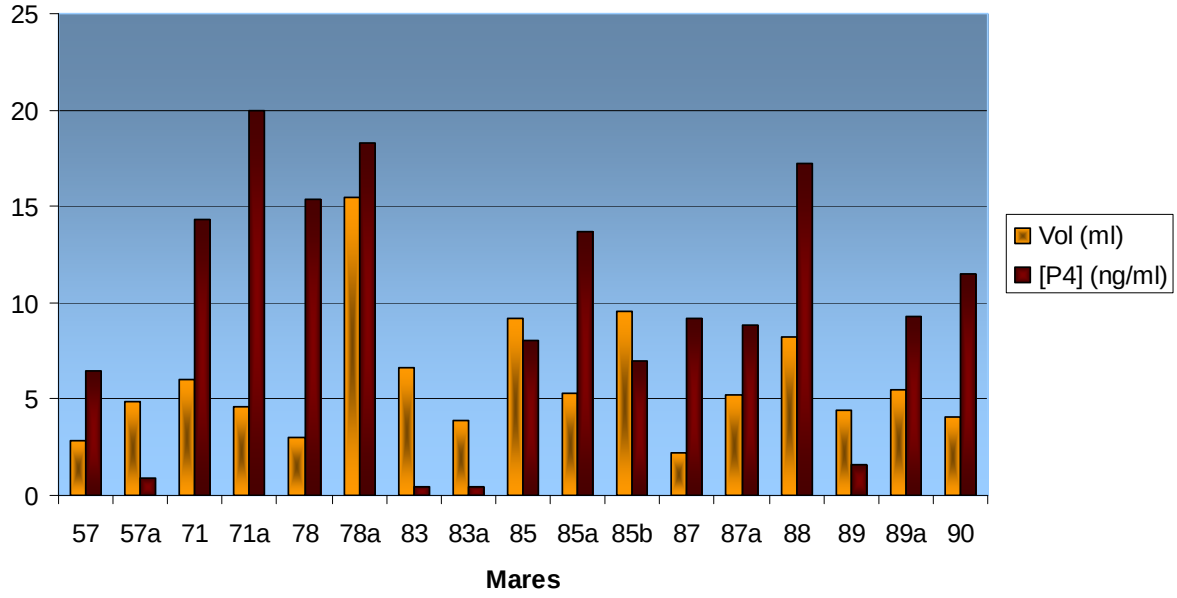
Graph 1 - Relationship between the cross diameters (D1, D2) (cm) and P4 (ng/ml) production.

Since the diameter of CL is one of most frequent parameters evaluated during the ultrasound exam and, it may reflect the CL extension, a relationship between those values and P4 concentrations was assessed (Graph.1). However, no significant correlation was found between these parameters (P>0.05). Also, no significant correlation was found between CL area and plasma P4 concentrations (Graph.2).



Graph 2 - Relationship between the area of CL (cm2) and P4 (ng/mL) production.

A relationship between the volume of the CL and progesterone was investigated. Nevertheless, no significant correlation was found between these parameters (P>0.05) (Graph.3).



Graph 3 - Relationship between the volume of the CL and P4 production.

After visual evaluation, no differences between the colour intensity and amount of pixels were verified among the samples.

Discussion

In the present study the concentrations of progesterone found during the luteal phase are in accordance with results described in prior works (Townson *et al.*, 1989; Bergfelt & Ginther, 1996; Ginther *et al.*, 2007a; Ginther *et al.*, 2007b). In three cases the concentrations of P4 were < 1ng/mL, which may denote that there was no functional corpus luteum. Since the time of ovulation was not determined, it was not possible to determine when these measurements were assessed. Due to the area of the CL, it is not very likely that those CL were corpora albicans

As it has already been done in some prior studies (Towmson *et al.*, 1989; Bergfelt *et al.*, 1996; Ginther *et al.*, 2007a), in the present work we assessed a possible relationship between the diameter and cross-sectional areas of CL and P4 concentrations. Bergfelt and Ginther (1996), described a parallel relation between the changes in the cross-sectional area of the primary CL and concentrations of progesterone in horse and pony mares during the early pregnancy. Ginther *et al.* (2007b) found a parallel relationship between the decrease of CL area and P4 concentrations during the luteolysis.

In the present study, since the day of ovulation was unknown, the stage of the luteal phase was unknown as well. So it was impossible to ascertain a relationship between the stage of the luteal phase and the progesterone production. However, in disagreement with those works, no correlation was found between the CL diameter and area with plasma P4 concentrations. Towson *et al.* (1989), found no differences in P4 production between two different CL types, with and without central nonechogenic area, and Arruda *et al.* (2001) did not find a relationship between CL size and P4 production in recipient mares, which may suggest that there are no correlation between the area , measured with US, and the P4 concentration.

Some authors tried to establish a relationship between the echogenicity of CL with P4 concentration in blood, since the gray scale values may reflect the luteal hemodynamics (Pierson & Ginther, 1985). Thus, Bergfelt & Ginther (1996), supported an inverse temporal relationship between the echogenicity of the CL and circulating concentrations of progesterone in horses and ponies mares. However, Arruda *et al.* (2001) described that an increase on CL morphoechogenicity is accompanied by an increase on circulating concentrations of P4.

In this study in order to evalute blood flow in the equine CL, a Power-Mode Doppler system was used, which has been previously described as having a higher sensitivity in depicting blood flow in small vessels when compared to the conventional Doppler technique (Rubin *et*

al., 1994). The vascular area was assessed but not quantified since at this time there was no software available to analyse the data.

Power Doppler measurements will be evaluated using a software *Program (Pixel Flux Scientific),* although after visual evaluation no differences between the amount of pixels could be noticed between active and non-active (based on P4 levels) luteal tissue. Those results contrast with previous works (Bollwein, 2002; Ginther *et al.*, 2007a). Bollwein *et al.* (2002) found that there was a high correlation (r=0,58; P<0.0001) between the cyclic changes of blood flow in the CL and plasma progesterone concentrations. In other work, Ginther *et al.* (2007a) found that the greater blood flow area was observed two days after the maximum progesterone concentration was detected, and that the rate of the decrease in CL blood-flow area was greater than the rate of the plasma progesterone decrease.

Since in this work only a few mares were followed for a short period, no relationship was assessed between the luteal phase, CL vascularisation, and P4 levels. Therefore further measurements should be carried out in order to fine-tune this technique in the horse. Measurements should be taken in mares during the whole luteal phase in order to give a more complete image of the possibilities of this technique, and CL vascular aspects during luteotropic and luteolytic phases, in the mare.

However, the difficulty on obtaining the same plane of scan on successive days, caused by the mobility of the loosely suspended ovary, may represent one limitation of this method.

Conclusion

In the present study no correlations were found between the CL cross diameters, area and volume with the plasma progesterone concentrations during the luteal phase of the mares.

The relation between the vascularised area, and the pixels in the power Doppler were not assessed because there was no software available to measure those parameters. Hopefully , this parameter will be evaluated *a posterior*. However in a subjective visual evaluation it seemed that no differences were found between the vascularised areas of CL and the respective P4 concentrations in blood.

It would be very desirable to evaluate individual mare's luteal variation. However, since sequential evaluations, as well as determination of ovulation time were not performed, it was not feasible to evaluate individual mares luteal variation.

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Annexes

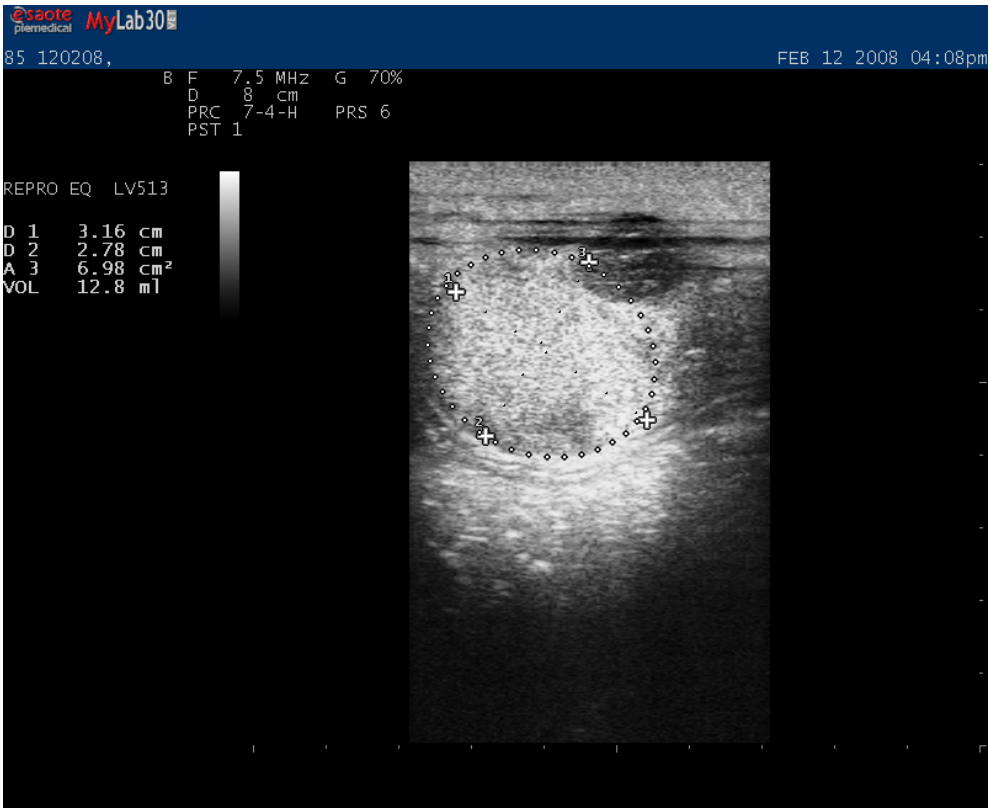

Fig 11 –CL measurements;