The ovine corpus luteum angiogenesis model: A tool for developing imaging technology

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Abstract—Robust tools for the quantitation of perfusion are not fully developed using contrast enhanced ultrasound (CEUS). The ovine corpus luteum (CL) is a transient gland in the ovary that is formed to produce the hormone progesterone essential for maintenance of pregnancy. Importantly, it has a dense microvascular network with predictable and well-regulated angiogenic mechanisms. In a number of different experiments it was shown that this property may be used to investigate and refine imaging methodology. Using a Philips iU22 ultrasound scanner (Philips Medical Systems Corp, Seattle, WA) in contrast imaging mode it was shown that a highly controlled experiment may produce high levels of reproducibility in the transit of contrast with standard uncertainty below 10%. Also, compartmental kinetics models were tested. The use of prostaglandin F2alpha promotes an intense anti-angiogenesis, allowing monitoring with CEUS prior to and following the demise of the CL microvasculature within 24 hours. Finally, the robust angiogenic property of the CL during the oestrous cycle allows further refinement of CEUS *in vivo*. In conclusion, the CL offers an attractive changing vascular bed for assessing existing and developing new clinically relevant perfusion imaging methodology.

I. Introduction

Advances in the noninvasive measurement of perfusion have been made in a number of human imaging modalities [1], but there is a consensus that the new techniques require further development [2]-[4]. The measurement of microvascular blood flow in various organs and in pathology has been the focus of Ultrasound contrast imaging research for over 25 years. The progress may appear slow and is attributed to the complexity of microbubbles as contrast materials, and the parallel evolution in transducer and signal processing technologies. Liver and heart pathology have demonstrated the usefulness of the modality in the clinic [5]-[7]. However, robust tools for the quantitation of contrast images are not fully developed, despite the fact that current ultrasound imaging is capable of detecting single microbubbles very sensitively [8]. In addition, current contrast imaging methodology achieves high sensitivity at even very low acoustic pressures [9]. It is important to note that *in vivo* research in the pursuit of accurate information on perfusion has a rather unsystematic character. On the other hand, there are several studies that use various disease models that affect the microvascular bed. For example microvascular density is a prognostic biomarker for a large number of cancers. Similar examples can be found in cardiovascular disease, inflammation or various interventional procedures such as transplantation. While a large number of feasibility studies justify further research, very little has been published on the inter- and intra- subject variabilities of these studies. PET with oxygen-15 labelled water is today the gold standard in clinical microvascular flow studies, due to its high sensitivity and breadth of quantitative information [11]. Quantification of myocardial perfusion with CEUS provided 32% intrasubject variability compared to 9% from PET [12].

To our knowledge an *in vivo* model of controlled progression and regression of angiogenesis for the development of microvascular imaging is not available and is much needed in order to harness the large number of variabilities encountered in the *in vivo* setting [10].

II. Basic Physiology of the Corpus Luteum

The corpus luteum (CL) is of key importance in the regulation of female fertility in mammals. It is a transient gland in the ovary that is formed after the egg is released and lasts for two weeks [13]. It is the most active gland in the body producing large amounts of the hormone progesterone. In humans, it is up to 2cm in diameter and has a blood supply, per unit mass, eight times that of the kidney. An intense development of new blood vessels is thus required to achieve this size in such a short period. In the absence of pregnancy the corpus luteum will undergo intense remodelling to lose its blood supply and disappear without scarring. It is this process of luteolysis that causes a woman to have a normal period. If the woman conceives a hormone (hCG) released from the embryo blocks this regression to maintain the structure, function and blood supply of the corpus luteum. Progesterone suppresses contraction of the uterus and maintains the pregnancy

While the importance of the corpus luteum is evident there are still unanswered questions on its function. The mechanism of its disappearance remains under investigated. It may give insight into the scarless healing seen in the ovary that has important implications for other body systems and tissue resilience, repair and replacement. Also, of wide significance is the process of angiogenesis that is important in cancer biology, inflammation and cardiovascular disease.

III. The Ovine Corpus Luteum in Ultrasound Contrast Imaging Development

All data were acquired from adult ewes under terminal general anaesthesia with all animal procedures approved and conducted in accordance with the Home Office Animals (Scientific Procedures) Act 1996 of the United Kingdom. The ovaries were exposed by laparotomy and clamped carefully to avoid interfering with the ovarian artery and vein blood

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supply. This enabled the ultrasound probe to be positioned and oriented for optimal data acquisition. Heart rate and blood pressure were monitored throughout the experiments.

B-mode and CEUS data were acquired using a Philips iU22 ultrasound scanner (Philips Medical Systems Corp, Seattle, WA) along with a linear array probe (either the L9-3 or the L12-5) operating on a contrast imaging mode (nonlinear pulsing schemes). The ultrasound contrast agent Sonovue® (Bracco, Geneva, Switzerland), approved for clinical use in Europe, was utilised. All scans were performed across the largest cross section of the CL. Data were stored for offline analysis using QLab software. At the end of the study the ovary was removed and fixed for immunohistological analysis of the microvasculature (lectin) for direct comparison with the ultrasonic image data.

A. *Compartmental kinetics*

In an initial study data from the CL (number of datasets *n=10*) and the human liver (*n=9*) were used to compare four indicator dilution models a. the lognormal function b. the gamma variate function c. the diffusion with drift models, and d. the lagged normal function, which have been used to model indicator dilution curves in different fields of medicine [14]. The focus of the study was to compare these in intravenous bolus injection protocols. The models fitted both sets of data well with the fit quality on the CL data being slightly better than on the liver metastasis data. In particular, the lognormal function and the diffusion with drift based models fitted the sub-regions of the time-intensity curve best, especially at the beginning of the curves where the rise in intensity with time is steep. All the models performed similarly, averaging $R^2 = 0.96$ for the CL data and $R^2 = 0.92$ for the liver data, when compared on entire data set performance. This may disguise their different performance at the start of the intensity curve, which is crucial in the measurement of Mean Transit Time (MTT) or Wash-in Time (WIT). In general, across the range of models the above difference in R^2 resulted in 50-500% increased uncertainty for the liver MTT compared to the CL.

B. *Echogenicity and Reproducibility*

In a subsequent study [16] the perfusion of the fully developed CL between days 8 and 12 of the oestrous cycle (*n=6*) was undertaken in order to establish a reproducible echogenicity [1]. This is the period where the CL has reached its angiogenic peak and is fully functional. Good contrast enhancement was observed in the CLs of all animals and the WIT averaged at 5.5 seconds with 9% uncertainty [16]. These uncertainties are generally better than others available in the clinical literature using a well controlled protocol [15]. Also, the animal CL WIT uncertainty between animals was 16% [16], which indicates a low variation between ovarian WIT between days 8 and 12 of the oestrous cycle. On another study the inter- and intra-animal dispersion was below 12% and 25% for time related paramaters such as the MTT and WIT, while intensity related parameters such as the area under the curve (AUC) and peak intensity had dispersions above 110% [17].

C. *Angiogenesis*

"The growth of the ovine CL is extremely rapid, linear from days 2-12, and primarily due to hyperplasia" [18] is associated with endothelial cell growth. This vascular growth has not been demonstrated using live imaging. A CEUS study only showed little variation of time parameters during the human oestrous cycle for the whole ovary [19]. However, no data are available on the CL specifically.

D. *Controlled Anti-angiogenesis*

While it is challenging to monitor a live animal for a number of days, the sheep CL model may be used for monitoring changes under vascular regression conditions. It is established that in sheep, the natural prostaglandin F2alpha (PG) is the causal agent released by the non-pregnant uterus which causes the CL to regress [20]. This is associated with a loss of endothelial cells over a 24h period [21] but this occurs after the decline in progesterone secretion.

Initial experiments were performed with 3 sheep in order to assess the usefulness of CEUS in this vascular regression [16]. The MTT provided a slight but not significant increase 24 hours after PG injection, while the AUC showed a significant decrease confirming the loss of vascular volume. These results were confirmed histologically with a significant reduction of the area of the lectin stained endothelium.

An initial study was performed with one animal in the first 6 hours after PG injection. An initial increase followed by a decline in AUC (Fig. 1) implies a more complex behaviour of the vascular bed, which requires further investigation. The AUC provided up to 40% standard error while the MTT error was less than 15%. No significant histological differences were found between 6 hours post-PG injection and control animals.

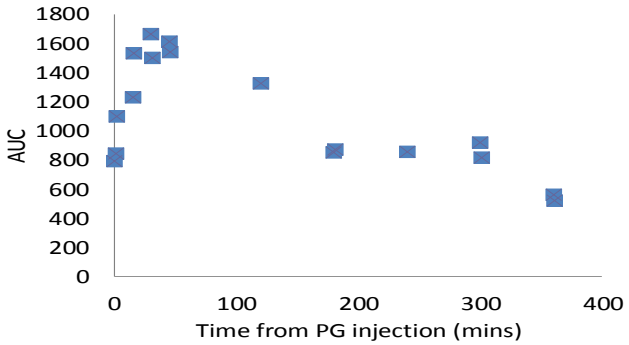

Figure 1. Area under the curve (AUC) in arbitrary units vs. time (min) from PG injection in minutes.

IV. Discussion

Large animal preclinical models encompass the use of human medical imaging methodology, which is a significant advantage in the development of new technology in disease relevant applications. This is largely because rodent medical imaging is different and less advanced.

A review of the initial results using the CL animal model of vascular regulation show the potential usefulness of this model in developing perfusion imaging methodology for CEUS and other imaging technologies. First, the well controlled experiment enabled a test of indicator kinetics models [14] providing highly significant fits [14],[16] and concluded on the suitability of different models by specifying respective strengths and weaknesses. The uncertainty of time related parameters such as WIT and MTT may be harnessed [16] to levels similar to the highly reproducible PET [12]. Imaging protocols were produced to achieve these results. On

the other hand AUC errors confirmed that intensity related variability requires further work to control [10].

Further the usefulness of this model lies in the ability to produce a vascular regression within one experimental day. Using parameters such as MTT and AUC to capture subtle vascular changes it is possible to test CEUS under conditions of changing vasculature. The complexity of such changes is encountered in all vascular related disease. Volume, flow, permeability and stiffness of the vascular bed change dynamically in disease and during treatment. Fig. 1) suggests that an initial vasodilation is followed by vasoconstriction in the first six hours post-PG injection. Vasoconstriction prior to apoptosis is a well established process, but not vasodilation [22]. Therefore it becomes apparent that the monitoring of blood flow and volume may provide information otherwise very difficult to collect using post-mortem techniques.

In general, the gold standard of endothelial cell area by means of histology as a measure of microvascular volume is not optimal. It is difficult to accurately provide histological data on the same slice of tissue that was scanned by the ultrasound field. There are also some fundamental concerns on such data. Although the endothelial cell area is related to vascular lumen cross section, the relationship is difficult to pin down. Further complexities are introduced when the tissue is excised and fixed, which would alter its mechanical state compared to that during the ultrasound measurements. These measurements remain the gold standard in the absence of other measurements, but should be used with caution as they provide large errors [23]. One of the challenges in future development of live imaging is to provide accurate means that allow the comparison with the results.

V. Conclusion

The ovine CL model provides a controlled and reproducible vascular regulation that may be assessed using clinical imaging equipment. Contrast enhanced ultrasound methodologies may develop further using this model as it provides a changing vascularity that simulates aspects of vascular changes in a number of disease models and related treatment strategies. Further work using the CL model may help address key limitations of CEUS.

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