Home Office

NON-TECHNICAL SUMMARY

Immunobiology of pregnancy in the mare in health and disease

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) Translational or applied research with one of the following aims:
  - (i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

reproduction, miscarriage, diagnosis, placenta

Animal types	Life stages
Horses	adult, pregnant
Ponies	adult, pregnant

Retrospective assessment

> The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Uses cats, dogs or equidae

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overarching goal of our research program is to improve fertility and reproductive health in mares through a better understanding of reproductive events such as conception and early pregnancy.

A retrospective assessment of these aims will be due by 04 October 2027

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve it's aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite continuous development of our knowledge in veterinary medicine, reproductive measures in mares remain unchanged over decades. Early pregnancy loss, defined as pregnancies that fail in the first two months after conception, is the greatest contributor to low reproductive outcome of mares. Knowledge on this period of pregnancy is still not well understood in horses. As a result, the diagnosis of the underlying cause is only possible in approximately 40% of cases making clinical management of the current and subsequent pregnancies challenging. Further, early pregnancy loss is associated with additional procedures for the mare and emotional and economic loss for the horses' owners, so any advances to reduce its occurrence has direct welfare benefits for the mare and the owner. An abnormal immune response to the fetus as well as underlying genetic variants key to embryonic survival and thus successful pregnancy are thought to contribute to pregnancy loss in other species, but knowledge of these processes and how they relate to pregnancy loss is limited in mares. Research on these key processes during critical periods of equine pregnancy will therefore ultimately leading to the development of new clinical management strategies, new therapies and diagnostic testing that could be applied to prevent such losses.

What outputs do you think you will see at the end of this project?

The causeThe cause of over 60% of cases of early pregnancy losses known remains unknown. Critical events during that time of pregnancy include the development of placenta, development of the key organs such as the heart and lungs and modulation of the mother's immune system. Results of this project will significantly increase (i) (i) the knowledge of key genes and pathways involved in placental development in mares including the first description of different types of specialised cells of the placenta;uterus and their role in guiding development of the horse placenta (ii) define the characteristics of equine immune cells in the uterus and their role in guiding development of the horse placenta (iii) identifying genetic variants (changes in the genome of the fetus) that are incompatible with life(changes in the genome of the fetus) that are incompatible with life. In the long term, identification of key proteins in cells in (i) and (ii) will inform future work that looks to understand the role of such pathways in pregnancy pathologies such as early pregnancy loss. The identification of genetic variants would result in the development of genetic tests that can be used to inform mating strategies that reduce the occurrence of pregnancy loss. Hence, this data would ultimately improve both the veterinary and breeding management of the mares. In addition, given the widespread role of immune cells in other physiological and pathological processes in the horse, comprehensive knowledge on the immune cells may inform further research in equine immunology. Our recent work on genetic variants that lead to early pregnancy loss in mares indicates that there may be some overlap in these pathologies between mares and women. Therefore, we also aim to use findings in mares to explore if the key pathways are described in early pregnancy loss in women and if not, design new studies to study their role in other species.

Who or what will benefit from these outputs, and how?

The beneficiaries of the results of this project are mares, mare owners, veterinary surgeons and the wider breeding industry. Results will provide new knowledge on the immune response and early development of the placenta and fetus in the equine pregnancy. This knowledge may help to explain pathology of reproductive disorders related to that period, such as early pregnancy loss. In turn, this may target better the veterinary and breeders care over the pregnant mare and hence reduce the incidents of the early pregnancy loss in this species. Moreover, chorionic gonadotropins which are secreted by equine placenta are also produced in human pregnancy. Thus, obtained results may be beneficial also for the human reproduction. In addition, immune cells which are involved in development of the placenta and changes to the immune system that allow the mother to tolerate the developing placenta may be important in other immune related processes hence the knowledge in this field may form groundwork for research in other fields of equine medicine. Similarly, large genetic variants have recently been described as a significant cause of early pregnancy loss in mares and it is expected that smaller variants such as loss or gain of small segments of chromosomes or single nucleotide polymorphisms will similarly contribute to the condition. Identification of these genotypes allows breeders to avoid certain matings that lead to lethal embryonic genotypes. The mare would benefit from avoiding a pregnancy loss, as when a mare loses her pregnancy, she requires an increase in veterinary procedures to investigate its underlying cause and then further procedures to establish pregnancy in a subsequent month. The owners would benefit as they are passionate about their animals and can emotionally suffer when their animals lose a pregnancy.

How will you look to maximise the outputs of this work?

Results will be presented at international conferences and published in leading journals in the field. This project is run as a cooperation between UK and Europe so there is an opportunity to share findings both locally in the UK as well as widely in Europe and Internationally. The investigators also regularly present at CPD events and clinical meetings (for veterinary surgeons) as well as to horse breeders with this new knowledge to also be shared via these channels as and when it becomes available.

Species and numbers of animals expected to be used

- Ponies: 20
- Horses: 15

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project aims to characterize pregnancy in the mare and hence the mares will be used as experimental animals. Anatomy of the placenta as well as the types of immune cells present at the maternal-fetal interface differs between species, for example mice have haemochorial placenta (only 2-3 layers between the fetal and mothers blood supplies) and gestation lasts 21 days while mares have epitheliochorial placenta (there are 6 layers between mother and fetuses blood supplies) with pregnancy lasting 340 days. The other species that have an epitheliochorial placenta are cow and sheep, but they also have a different shaped placenta. Hence, mouse or ruminants cannot be used as a model for equine pregnancy under most situations. Moreover, this project will primarily benefit horses thus it is the species used in the project. To determine the immune mechanism of placental development, samples will be taken during important timepoints of the placenta formation that around the time of fetal cells invade in the mothers' tissue and once placental formation is completed. To identify the exact period when genetic variants can lead to lethality or a compromised fetus, conceptuses will be recovered throughout the early period between days 6 (when the blastocyst can be recovered from the uterus and day 65 corresponding to the window when pregnancies are most likely to be clinically lost) to compare normal developmental features to those features of clinical cases of early pregnancy loss.

Typically, what will be done to an animal used in your project?

Samples will be collected from clinically healthy mares over the age of 2 years. There are three sets of procedure that will be used. Protocol 1. Pregnancy will be established in mares using standard breeding procedures for artificial insemination. Under protocol 2, once pregnancy is confirmed, the embryo will be flushed out of the uterus, blood samples obtained and a biopsy of the endometrium obtained at various time points after conception (using equipment used to routinely biopsy mares in clinical practice). For a subset of mares, we will also place a probe through the cervix (non-invasive) to measure oxygen levels. This will be removed as soon as the reading is recorded, normally after 10

minutes. In Protocol 1, some mares will have a mock pregnancy. That is mares will be monitored for ovulation then receive a hormone daily after ovulation that mimics the hormonal state of pregnancy. These control mares will also go onto P2, where they have similar procedures (biopsy of endometrium, blood sampling) except they do not have a uterine flush for recovery of an embryo. For a subset of mares, we will also place a probe through the cervix (non-invasive) to measure oxygen levels. Under protocol 3, we will use non-pregnant mares, usually over the winter, where blood samples will be collected from mares usually whilst remaining in the field to be used to isolate white blood cells for laboratory based experiments. This procedure is typically completed in under 5 minutes and the mares usual grazing is minimally disrupted. If we want the blood samples to be collected from a mare at a specific stage of her cycle, we may also administer standard hormones (used in breeding mares across the UK) prior to blood collection to mimic the hormonal environment such as progesterone of the pregnant state.

Prior to entering the project all mares will be examined by a veterinary surgeon and ultrasound examination of the uterus and ovaries will be performed to confirm their health status. They will be brought into the stocks in advance of commencement of procedures to acclimatise them to the space where the majority of procedures will be performed and a positive experience offered such as the use of food whilst in stocks. To establish pregnancy, mares will restrained in stocks and monitored by transrectal ultrasonography (similar to the management of breeding mares) and when the clinical findings indicate impending ovulation, the mares will be inseminated with commercially available cooled semen using standard veterinary procedures. As controls, some mares will either receive injections of a hormone, oxytocin (licensed and routinely used in clinical practice), or altrenogest orally in order to prolong the life of structure on the ovaries that produces the hormone of pregnancy called progesterone, in order to mimic a pregnancy. Pregnancy will be determined and monitored by transrectal ultrasound of the mare. At 6 to 65 days after establishment of the pregnancy, the mare will be sedated and biopsy samples of the endometrium (using biopsy forceps) and blood samples from the jugular vein will be collected. The conceptus will be recovered following non-invasive uterine lavage. Endometrial biopsies and blood samples will be collected from the control mares in the same days after ovulation also under sedation. After sample collection all mares will be administered a medication in order to stop progesterone production and return the mare to oestrous. In the consecutive cycle mares may be returned to P1, and will be again monitored daily with the ultrasound and at the appropriate day of the cycle, will be inseminated again or receive medications in order to act as a control. Each mare will have no more than three control cycle/embryo recoveries in any one year under P2. Before every collection of the endometrial samples and embryos, mares will be sedated. In the case of post-breeding endometritis, (a relatively common condition in the mare, that can be detected through a clinical examination of the uterus but not by a change in behaviour), will be treated with a medication to clear fluid. After all procedures in the stocks, mares will be returned to the field. In our experience, they are usually back grazing immediately after rectal examinations and after sedation, within 10-60 min of the procedure being completed.

What are the expected impacts and/or adverse effects for the animals during your project?

The main impact will be some transient discomfort at the time of embryo recovery, although due to sedation and our previous experience this is predicted to be mild. Mares are expected to be returned to their fields and grazing very shortly after procedures and no change in behaviour has been noted over the previous 10 years performing these procedures.

Some mares may experience a transient (1-2 days) inflammation of the endometrium. This level of inflammation causes no detectable discomfort in the mares (in the majority of cases with this condition, they show normal feeding and paddock behaviour) but can impact the fertility of the mare so mares will be monitored by examination of the uterus by ultrasonography after the procedures to ensure if they do experience this transient inflammation, it is treated.

There are possible other adverse effects from some of the hormones we use, but such effects are transient (10 minutes to one hour), mild (sweating, mild abdominal discomfort) and uncommon (between 1:100 to 1:1000 mares) so are likely to not be experienced with the number of mares used in this project. Horses subjected to rectal examination can experience a tear to the rectum. This is incredibly rare though, estimated to be 1:5000 - 1:10 000 horses. Precautions are made to minimise these risks such as use of previously trained staff, training of new staff and good restraint of the mare.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All Planned procedures within the project are of mild to (low) moderate severity and will be performed in horses and ponies. Over a 5 year period, we expect to use between 25-30 mares.

What will happen to animals at the end of this project?

- Rehomed
- Kept alive
- Killed

A retrospective assessment of these predicted harms will be due by 04 October 2027

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Currently there are no in vitro models to study equine pregnancy, specifically maternal immune response to the developing fetus nor the complex development of the placenta. Immunomodulatory signals by which the conceptus influences maternal cells remain unknown in a horse thus it is not possible to design suitable in vitro models. Moreover, functioning of the maternal immune system is a part of physiological changes of many body systems which influence each other in response to

pregnancy and due its complexity can’t be replaced by an in vitro model. In order to confirm genotypes associated with lethality, it is important to show that they are absent in pregnancies that are viable and also characterize the associated genes and proteins impacted by the variant in normal equine conceptuses – thus requiring a whole animal.

Which non-animal alternatives did you consider for use in this project?

Reproductive tissues obtained from the abattoir and our bank of archived tissue remaining from previous studies can supplement aspects of this work but not the delivery of all objectives. We also looked for cell lines but these are currently not developed for the placental cells or the immune cells in the horse.

Why were they not suitable?

As we have not as yet established the phenotype of the immune cells at the maternal-fetal interface, alternative non-animal models are not possible. Once we establish the phenotype of important cell populations, we could then model the interactions between the immune cells and endometrial cells using in vitro models established using ex vivo tissue from abattoir tracts and new systems such as organoids along with white blood cells isolated from peripheral blood. We did consider the use of abattoir material, and indeed that has been used for optimization of the assays but this was deemed only of use once we had a better understanding of the cell types and is unable to provide pregnancy tissues as pregnant mares are not sent to slaughter. We have used conceptuses from clinical cases of early pregnancy loss that would otherwise be discarded by the veterinary surgeon to generate our preliminary data for genetic experiments. The next step is to verify the role of the genes we have discovered using normal conceptuses. We can use some tissues archived in previous projects but such tissues do not include all time points needed to verify the impact of the variant under investigation. Further conceptus material in early development is key to understand the expression of proteins key to early pregnancy.

A retrospective assessment of replacement will be due by 04 October 2027

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will primarily use ponies for the experiments as they can be best acclimatised to the procedures and be easily handled. We may also use horses (primarily Thoroughbreds) for some experiments when the genetic background is of particular importance for the experiment.

We have estimated the number of animals to be used based on (i) our experimental design and the number replicates required to obtain statistically significant results or describe gene and protein expression in the equine conceptus and (ii) our knowledge of reproductive efficiency in mares including conception rates per cycle and the common reproductive conditions that might impact the fertility of mares and thus conception per cycle.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We did review the NC3Rs experimental design assistant but it was deemed unsuitable for these particular projects where we are utilizing ex vivo tissues for in vitro experiments. Therefore, we have focused on the design of the in vitro experiments.

Thorough review of the available literature has been performed and based on other studies with the use of scRNA sequencing the minimal number of samples and hence animals which would bring significant results was determined. Because the procedures planned within the project are of mild severity every mare will be used both as a pregnant and control animal. This approach reduces the number of mares.

For example, for our immune response experiments, samples will be collected from 4 biological replicates (4 mares). The reported minimal technical variation of the provider of scRNAseq and published studies where scRNAseq was used on human placenta supports the use of 4 replicates per time point for this experiment. Samples will be taken at 4 time points (two stages of pregnancy and then control samples for each stage) meaning a total of 16 samples. As each mare will act as its own control, we would expect each mare to contribute a control and pregnancy so would need 8 mares. Even with efficient veterinary care a mare may not become pregnant during every single reproductive cycle. In our experience and in line with published data, the chances of conception per cycle is 60% and mares can only be bred over the breeding season (spring to early autumn). Further, also some mares will enter a period of prolonged abnormal cyclicity due to dioestrus ovulations or haemorrhagic anovulatory follicles reducing fertility. Therefore, two additional mares will be monitored as part of the herd so the most fertile 8 mares can be used for the procedure at any one time. For the genetic experiments, our published experiments suggest that n=3-5 (pending on the specific gene/protein being studied) provides sufficient power to detect differences in gene expression across pregnancy, demonstrate proteins are expressed in tissues at the time point of interest. We plan to collect samples at three stages in development and therefore need 15 collections. Similar to the calculations above, we expect 10 mares to be necessary to provide the conceptus material.

The ARRIVE guidelines have also been consulted.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All mares used in the experiment will be clinically healthy and the pregnancies will be established according to the current veterinary protocol to maximize the probability of establishing pregnancy during

one cycle. It will not reduce the total number of the animals, however, it may reduce the number of procedures performed on every mare in order to collect samples needed. We will utilize archived tissue samples where possible. We will also continue to obtain tissue samples from dead pregnant mares submitted by veterinary surgeons under owner consent. These are mares that die/ are euthanased whilst pregnant following a life terminating condition such as severe colic or fracture to a limb but otherwise have been healthy and to be carrying a clinically normal pregnancy immediately prior to the death. These will provide tissues that will be used in two ways: to obtain stages of pregnancy beyond 70 days that are not generated as part of the project licence and also if the gestational age is less than 70 days, to supplement the tissues generated as part of this licence (ie directly reducing the number of procedures we do).

A retrospective assessment of reduction will be due by 04 October 2027

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The main methods to be used are blood sampling, establishment of pregnancy using standard veterinary procedures and flushing of the uterus and endometrial biopsies using standard veterinary procedures. The horses will be housed outdoors in paddocks under conditions very similar to companion animals and stimulation provided through the interactions both with other horses (housed in a herd) and people. The mares will be given an opportunity to acclimatize both to the stocks and people prior to initiating any procedures. This will involve hand feeding in the stocks to provide a positive experience. Conceptus recovery will be performed at early stages of pregnancy (first 2 months of an 11 months gestation). At this stage of gestation, the conceptus is easily dislodged and can be recovered via a catheter placed in through the cervix into the uterus. This non-surgical method of trophoblast recovery is the least severe method available to obtain trophoblast cells. All aspects of this procedure are also carried out in clinical practice for either establishment of pregnancy or in embryo transfer programmes and complications associated with the procedures are rare. Endometrial biopsies will be performed in stocks to minimize the risk of rectal tear. Both the embryo flush procedure and biopsy will be performed under sedation to minimise any stress experienced by the mare.

Why can’t you use animals that are less sentient?

This project is important for equine reproductive health hence mares must be used to establish key cells and proteins involved in early development. Placentation differs notably between species and hence immune mechanisms facilitating this process as well as maternal tolerance. For example mice have haemochorial placenta and pregnancy lasting 21 days whereas placenta in horses is epitheliochorial with pregnancy around 340 days. Thus the mouse can not be used as a model species for horses.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The proposed in vivo studies is the most refined available to study pregnancy in the horse, as the alternative would be to obtain trophoblast and endometrial samples surgically from pregnant mares, a procedure that would require general anaesthesia or euthanasia and a more significant impact on the animal. These less invasive procedures have been regularly performed by the investigators originally clinically in practice and over the last 15 years in research animals. Therefore, many of the refinements have already been introduced for the proposed procedures through previous experience (for example, monitoring of endometrial health independent of behavioural changes in the mare, use of reproductive hormones to minimize the number of oestrous cycles required to establish pregnancy). We propose to add the use flunixin immediately after the endometrial biopsies to ensure any potential pain or discomfort we might not detect through behavioural changes is prevented by reducing inflammation. All mares are already regularly monitored daily for their response to procedures and should any complications occur, monitoring increased further as required.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines that are intended to improve the reporting of research using animals and maximise information published as well as minimise unnecessary studies. These are followed to ensure we record appropriate information as part of the project, to audit methodologies and ensure, when publishing, we report the methodology and mare characteristics arising correctly.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The researchers will continue to attend lectures provided by the NC3Rs representative and also review the literature to ensure high quality reproductive care (and hence the greatest efficiency and least procedures) is achieved to overall reduce procedures. The website of the NC3R’s will be followed throughout the project duration to keep up to date with new developments. The applicant and team regularly attend and present at both national and international clinical equine reproduction conferences allowing them to remain up to date on best practices for breeding mares.

A retrospective assessment of refinement will be due by 04 October 2027

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?