A Molecular Study On Hemorrhagic Anovulatory Follicles

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This study investigates gene and hormone levels in HAFs, the leading cause of ovulation failure in mares, using a novel technique (Follicle Wall Biopsy-Trinity) to collect multiple follicular samples.

In mares, hemorrhagic anovulatory follicle (HAF) syndrome is one of leading causes of ovulation failure and consequent subfertility. During a normal reproductive cycle, a single follicle (the structure that holds and nurses the egg) will grow until ovulation, upon which the structure will burst and release an egg, with the potential of becoming fertilized to form an embryo. For animals that experience HAF syndrome, a follicle that would normally ovulate will instead accumulate blood and expand to form a large structure that persists in the ovary for an extended period of time, until it naturally regresses after two to three weeks. As such, presence of HAFs leads to complete loss of the reproductive cycle for which it is present, since fertilization cannot be successfully achieved unless an egg is released.

For animals used for breeding in industries like showing and racing, this has severe financial implications as timing of reproduction, including insemination and birth, is often tightly controlled to produce high-quality foals as early in the year as possible. In the Thoroughbred industry alone, over 25,000 mares were bred, 17,000 registered foals were born, and the average yearling sold for over $80,000 in the year 2023. As these numbers show, this industry is a high-volume, high-value, and fast paced market where even a single missed reproductive cycle can have detrimental and long-term impacts. While incidence of this syndrome in mares ranges from approximately 5% during the early breeding season to 25% in the late breeding season, other factors such as age, administration of certain drugs, and mare predisposition to this syndrome also greatly affect the incidence of HAFs.

Previous research has focused on characterizing HAFs versus normally ovulating follicles, evaluating risk factors for HAF formation, and development of injections into the follicle as a potential treatment for HAFs. However, no study to date has explored the differences in hormone and gene expression of key factors in HAFs versus normally ovulating follicles to see if the egg and the follicle as a whole can be rescued from anovulation.

To answer these key questions, the current study will use a new, cutting-edge technique to collect several types of samples (tissue from the follicle wall, follicular fluid, granulosa cells, and the egg) from induced HAFs and normally ovulating follicles. To investigate the molecular differences between HAFs and normally ovulating follicles, we will examine various hormones, growth factors, and cell markers to see if there is any potential to save the egg cells or the whole follicle from the negative effects of HAF formation.

We predict that while certain negative markers, like cell death and inflammatory markers, will be higher in HAFs, the follicle itself will be more affected than egg, meaning that perhaps with proper treatment, the follicle can be saved, and the egg may still be fertilizable upon ovulation.

To test these hypotheses, we have designed three experimental groups:
(1) Negative Control Group, with mares that are allowed a natural reproductive cycle;

(2) Positive Control Group, with mares that have an ovulation induced via chorionic gonadotropin (hCG);

(3) HAF Group, with mares that will have HAF formation induced via an already well-established protocol.

When a mare's follicle becomes large enough, she may be given drugs, and samples will be collected 38 hours later. For this study, we will be collecting six different types of samples: (i) blood, to measure hormone levels, (ii) follicular fluid, to measure hormone levels and markers of ovulation, (iii) follicle wall tissue, to measure expression of growth factor and hormone receptors, inflammatory and cell death markers, and enzymes related to ovulation, (iv) mural granulosa cells (the cells lining the follicle wall), (v) cumulus granulosa cells (the nurse cells surrounding the egg), and (vi) the egg, to measure levels of hormone receptors, cell proliferation markers, and cell death genes in the three

aforementioned sample types. To collect these samples, we propose the use of a new, cutting-edge technique that allows for the collection of all six of these samples in the same procedure.

This technique, called Follicle Wall Biopsy-Trinity (FWB-Trinity), recently designed and developed by our lab, ensures that a mare will only have to undergo a single invasive procedure per reproductive cycle, serving to maximize animal welfare and minimize animal use. Furthermore, preliminary studies generated by our lab show that this technique can be performed multiple times on the same mare, allowing for repeated sample collections over time. In summary, by performing this project, we will be able to provide a foundational study on HAFs, with the hope of advancing knowledge on this syndrome and to provide groundwork for eventual development of a treatment.

Importance to the Equine Industry: For mares used for breeding in industries like showing and racing, every reproductive cycle is precious, as timing of reproduction, including insemination and birth, is often tightly controlled. However, every breeding season, thousands of mares experience fertility problems, often linked to aspects of genetic predisposition and intensive management practices.

In cases of anovulation, where a mare's follicle fails to ovulate and release a fertilizable egg, the presence of a hemorrhagic anovulatory follicle (HAF) is the leading cause of subfertility. In the Thoroughbred industry alone, over 25,000 mares were bred, 17,000 registered foals were born, and the average yearling sold for over $80,000 in the year 2023. As these numbers show, this industry is a high-volume, high value, and fast paced market where even a single missed reproductive cycle can have detrimental and long-term impacts.

Furthermore, in other breed industries like Quarter Horses and Arabians, practices such as ovum pick-up and intracytoplasmic sperm injection are becoming standard; however, the effects of HAFs on the egg cell have not been investigated. By examining the molecular differences between HAFs compared to normally ovulating follicles, we can determine if there is potential to save the follicle from anovulation and rescue these eggs. However, previous methods for collecting follicular samples have involved a mare undergoing multiple invasive treatments in a short time. Thus, this study will use a novel technique developed in our lab, Follicle Wall Biopsy-Trinity, to collect several different types of follicular samples in a single procedure, serving to maximize animal welfare and minimize animal use.

In summary, by performing this project, we will be able to provide a foundational study on HAFs, with the hope of advancing knowledge on this syndrome and to provide groundwork for eventual development of a treatment.

Funded by Grayson-Jockey Club Research Foundation