Ovarian protein hormones as biomarkers of fertility in dairy cows – Is it an acceptable model to predict infertility in dairy breeds?

By

Ruben Lopez-Carrillo Jr

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Ruben Lopez-Carrillo Jr

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ABSTRACT

Female dairy cattle are particularly prone to infertility/subfertility with pregnancy rates ranging as low as 27% to a high 40%. On average it takes more than one breeding/insemination event for dairy cattle to become pregnant. This results in a loss of the number of calves a female would have per year. There have been different methods that producers use to ensure a female ovulates in a timely fashion during artificial insemination (AI), including synchronization of estrus and ovulation by use of exogenous hormones. However, it is difficult to ascertain if a female is experiencing a fertile cycle at time of breeding. The purpose of this study was to determine if the ovarian protein hormones anti-Mullerian hormone (AMH) and inhibin A can be used as biomarkers for fertility of dairy breed cows. Blood was sampled from dairy breed mature cows and first-calf heifers housed at the Oregon State University Dairy before and during breeding by timed artificial insemination techniques near the time of insemination. Their serum was analyzed for AMH and inhibin A concentrations by Enzyme-Linked Immunosorbent Assays (ELISAs) previously validated in cattle (Ansh Labs) to determine if changes in these hormones correlated to pregnancy outcome. Samples were assigned to assay runs at random. Inter- and intra-assay coefficients of variation (CV) were within acceptable limits for ELISAs (AMH 17.4% and 2.8-2.7%; inhibin A 7.8% and 5.1-3.6%). While inhibin A assays had lower inter-assay CV, even with randomizing samples to two separate assay runs, significant differences were detected between samples run on different days (students t-test p=0.03). This might reflect a skew in pregnant females not detected in AMH assays (p > 0.9; n=45 samples, 27 non-pregnant, 18 pregnant), however, to avoid assay bias a Mixed Model function of SAS was used to analyze serum inhibin A results, using assay as a random variable. AMH levels were analyzed by one-way ANOVA (SAS). Levels of AMH within one week of breeding (pre-breeding sample, n=27; 17 non-pregnant, 10 pregnant), or on day of AI (n=17; 9 non-pregnant, 8 pregnant) were not different in females by pregnancy status (p’s > 0.3, 0.5). Similarly, serum levels of inhibin A were not significant different by pregnancy status when analyzed pre-breeding (p=0.5), however on the day of AI there was a weak tendency (p=0.19) for females who did not become pregnant to have lower levels of inhibin A (3.4 ±3.2 vs 5.6±3.3). Unfortunately, a limitation of the current study was the low number of females analyzed on day of AI, due to a combination of factors including availability of trained AI technicians and environmental factors during the study period at the OSU Dairy (July-September 2021). Inhibin A is produced by the dominant follicle and corpus luteum (CL), and higher levels may indicate a better-quality follicle is present on the ovary capable of sustaining a pregnancy. Further
research is needed into possible utilization of inhibin A as a biomarker of pregnancy success in dairy cows.

INTRODUCTION

As mentioned by F. López-Gatius, the reproductive performances of dairy cattle over the past few years has not improved significantly [3], with pregnancy rates ranging as low as 27% to a high 40% [27]. In fact, reproductive disorders have significantly developed increasing infertility/subfertility [3] globally because dairy herds have intensely focused on improving milk yield and conformation traits instead of genetically improving fertility traits since it is a lot easier to measure and directly connected to profitability [2][9]. Dairy farmers want to have the best milk production traits as one can expect but it is not that simple to choose for the best because there is a negative relationship between milk and fertility traits [2]. Selecting for the best milk yield and fertility traits is difficult because of the pleiotropic effect of genes for production and fertility where identical genes underlie expression of these traits [2]. Even though fertility has low heritability, there is adequate additive genetic variation to allow for genetic improvements by selection [2].

Other major factors that have been found to be associated with poor fertility include inappropriate management and environmental/climatic conditions [8][2]. A genotype for one breed may not work as efficiently as another breed regarding the environmental conditions they are put under [8]. Deciding the best health and management methods for fertility begins with understanding in which environments each herd’s genotype results in the best reproduction rates [8]. Hot temperatures for instance are an environment that decline fertility in cattle because heat stress has a negative effect on oocytes and early embryos [10]. Although environment and management are two of the most significant influencers on fertility, one must not forget about looking into other key factors like proper nutrition, herd type, time, and type of breeding [1] lactation number, calving year and calving season known to also affect fertility [9].
There are three methods used in cattle reproduction today that many producers practice, which include natural mating, embryo transfer, and artificial insemination. Although there has been a lot of progress using these methods, preserving fertility has still been difficult since these reproductive technologies and natural mating practices haven’t necessarily improved the reproductive fulfillment of fertility gradually [3]. They only have allowed for better timing of breeding using estrus synchronization and at times improving repeat breeder cattle conception rates [3] [11]. Estrus synchronization involves manipulating the luteal phase of dairy cattle by either shortening or lengthening the cycle using exogenous hormones which helps producers in understanding their herds calving interval, scheduling calving seasons, milk availability and proper use of artificial insemination technique. [13] [14]. It is used in all three breeding practices today.

The most common practice of reproduction for dairy cattle of all three has been artificial insemination (A.I.), containing the best and most rapid dispersal of genetics for dairy farmers [6]. A.I has been successful in dairy cattle due to the technological improvements in semen as well as the positive correlation to cattle favoring these genetic modifications [6]. In industry, A.I has been victorious due to its simplicity and affordable use compared to embryo transfers and natural mating [6].

Embryo transfer (ET) in dairy cattle refers to the process of producing a genetically superior embryo from a donor cow at an early age that can then be placed into a recipient cow [7]. This practice is becoming more common today with cattle but it’s much more expensive to use ranging around $165 for the transfer of one embryo that may or may not develop into a viable pregnancy [7]. ET has been found to improve conception rates in repeat breeder cattle [11]. Repeat breeding is a reproductive disorder in dairy cattle who did not become pregnant after three or more breeding attempts [11] [12].

Artificial insemination and embryo transfer are more likely to be successful if done in a cycle of a cow that has a high probability of fertility. Unfortunately, fertility success is impacted by several factors, and many scientists are working to identify one which might have the highest likelihood of correlating with fertility. This would be known as a biomarker that could guide producers' decisions. Previous studies have been done to see if we can use reproductive hormones as a potential biomarker for fertility. Anti-Mullerian hormone (AMH) for instance, is a hormone in dairy cattle that is positively/directly correlated with antral follicle counts [15][18]. It is produced by granulosa cells from early antral follicles within
the ovary where it then enters circulation, allowing us to detect and measure it through enzyme-linked immunosorbent assays (ELISA’s) using blood samples [16]. AMH is produced at its highest concentrations levels in primordial, primary, and secondary follicles [17]. It then decreases in concentrations at the dominant follicle stage [17]. Batista et al. [18] found that heifers who had lower circulating AMH had larger follicles towards the end of their cycle with much greater likelihood of ovulation, compared to those with greater circulating AMH. Essentially, AMH might be a good hormone to analyze and see if this could be used to predict individual cow’s possibility of pregnancy, making this hormone promising in fertility-based studies.

Another hormone produced during follicular growth is Inhibin. Inhibin is a dimeric protein hormone synthesized by follicular granulosa cells [19]. There are two different forms of Inhibin which are Inhibin A and Inhibin B. Inhibin B promotes follicle stimulating hormone action in nondominant follicles during the preovulatory period, ensuring the development of a single dominant ovulatory follicle [21]. It is found during early antral follicle development similar to AMH [12].

Inhibin A is an isoform produced by dominant follicles in the corpus luteum. Data suggest that a dominant follicle promotes high levels of inhibin A as the follicle progresses of dominance through ovulation and does not fall until after ovulation [20]. Analyzing inhibin A will essentially give us more information on the cycle and the status of the dominant follicle. It is a growing area of research when it comes to fertility.

Diagnosis of ovarian dysfunction can be time-consuming for the dairy industry. AMH and Inhibin are two good potential biomarkers in the class of reproductive hormones to study regarding fertility.

The objective of this study was to analyze AMH and Inhibin A concentrations before and during breeding to determine if levels of these serum hormones correlate with pregnancy outcomes. The hypothesis was that Inhibin A may give us more information than AMH itself.

MATERIAL AND METHODS

Twenty-seven female Jersey dairy cows (Bos taurus) housed at Oregon State University in Corvallis Oregon (OSU) out in the dairy farm ranging in different age groups (mature cows and first-calf heifers), were used to complete this study.
All studies were approved by OSU’s Institutional Animal Care and Use Committee (IACUC) prior to study initiation. We used 27 female dairy cows (Jersey & New Zealand breeds) that were housed in a dairy shed and had access to go out and graze into the pasture mainly each day. All being used in the study were lactating cows being milked twice a day in the milking parlor just a few feet away from the shed. (4:00AM & 4:00PM).

OSU’s Dairy farm student workers and professional staff managed husbandry for them throughout the study providing feed, water, management, etc. Many of these females were part of the milking herd at the time so they were monitored several times per day by milking staff. Dairy center staff were notified of animals who are under protocol or had recent blood draws to allow for close monitoring of site of blood draws, and research staff (myself) who drew the samples monitored the females once daily for up to 1-week post-sampling. Sites were observed visually for signs of swelling and bruising in case further treatment was needed.

We took a blood sample approximately 1 week before and at the time of artificial insemination/AI (breeding) so that we could compare different hormone levels and determine how they fluctuated during the different stages of the cycle. Precise detection of heat on each cow was done using the OSU dairy staff and additionally the cows had pedometers on one foot that activated when more than the usual activity/movement was done by each cow signifying potential heat. At the end of the sampling period a total of 45 blood samples were drawn for this study. We obtained samples from n= 27 cows: Pre-breeding sample n=27; 17 non-pregnant, 10 pregnant, and on day of AI n=17; 9 non-pregnant, 8 pregnant.

Blood samples:

Females were restrained by headlocks for sample collection. The tail was raised vertically with one hand until horizontal with the ground and at approximately 150mm from the base of the tail, the groove lying in the ventral midline of the tail was located. The area where blood draw was to be done was always swabbed with 70% alcohol-soaked cotton ball beforehand. After, midway along the body of a coccygeal vertebra, the vacuum needle was inserted perpendicularly to the surface of the skin to a depth of a few millimeters while at the same time inserting the vacuum tube (BD Vacutainer re-top tube, no anticoagulant or preservative) so that the blood can fill into the tube. Cattle are estimated to have a total blood volume of 27,000-36,000 ml (52-60 ml/kg body weight), so 5 ml of sample, compared to 27,000 ml is ~0.02% of total blood
volume. Once the sample was taken, pressure was applied to the venipuncture site until the bleeding stopped. The total amount of blood drawn from each cow for 1 estrus cycle was ~10 ml.

Samples were identified by cow and date, and were maintained throughout the study as separate samples. The samples were then taken to be refrigerated overnight (at 4ºC) so that they would clot, and the serum could be removed by centrifugation the following day (2,500 rpm, Beckman Allegra X-22 R Tabletop Centrifuge). When centrifuging was complete, we collected the serum and froze each sample at -20ºC until day of assay.

Enzyme-Linked Immunosorbent Assays to Quantify AMH and Inhibin A in Serum

Quantifying both AMH and Inhibin A concentrations in cattle serum was done by using an enzyme-linked immunosorbent assay (ELISA Ansh Labs, Bovine AMH Elisa & Equine/Canine/rodent Inhibin A ELISA). A sandwich ELISA was used which involves two antibodies detecting different epitopes on the same antigen. The first antibody is coated on the surface of the multi-well plate and is used as a capture antibody to facilitate the immobilization of the antigen (AMH or Inhibin A). The second antibody is conjugated and detects the antigen.

Four separate assays were run on different days with similar procedures followed for each assay. For each assay procedure we only used half of a plate (48 wells) and not the entire plate (96 wells). Samples were obtained from the freezer and thawed on ice until they reached room temperature before beginning each ELISA assay.

Each ELISA was performed as suggested by the manufacturer. The known concentrations of antigens (calibrators), as well as controls, and unknown samples were added to individual wells containing antibodies specific to either AMH or Inhibin A. Depending on the type of assay, we then added buffers (AMH assay buffer or Inhibin A assay buffer) followed by placement on a microplate shaker (600-800 rpm) for 120/150 minutes. The microplate was then flushed with a wash solution. Next, the biotin-conjugated secondary antibody was added into each well and incubated on the microplate shaker for 60 minutes. To prevent detection of non-specific reactions the plates were again flushed several times with a buffer. Finally, the streptavidin enzyme conjugate was added to detect the bound antigen. Following more washing to remove unbound conjugate, colorimetric detection was
performed by the addition of tetramethylbenzidine chromogen, the substrate for the enzyme. To stop the enzymatic reaction, the stop solution (0.2M sulfuric acid) was added and the plate was analyzed immediately after.

The iMark™ Microplate Absorbance Reader was used to read each assay, set at a wavelength of 450 nm and the optical density recorded. The data were then transferred to excel manually and analyzed using OriginPro 2019. Each Inhibin A and AMH assay kit came with known concentrations of the used calibrators which we used to compare to optical density and created a standard curve using a cubic regression curve fit function. Inhibin A calibrator concentrations ranged from 0 pg/ml – 700.0 pg/ml with quality control at 66.0 pg/ml – 185.0 pg/ml. AMH calibrator concentrations ranged from 0 pg/ml – 2116.0 pg/ml with quality controls at 288.0 pg/ml – 1030 pg/ml.

STATISTICAL ANALYSIS

Samples were assigned to assay runs at random. Inter- and intra-assay coefficients of variation (CV) were within acceptable limits for ELISAs (AMH 17.4% and 2.8-2.7%; inhibin A 7.8% and 5.1-3.6%). While inhibin A assays had lower inter-assay CV, even with randomizing samples to two separate assay runs, significant differences were detected between samples run on different days (students t-test p=0.03). This might reflect a skew in pregnant females not detected in AMH assays (p > 0.9; n=45 samples, 27 non-pregnant, 18 pregnant), however, to avoid assay bias a Mixed Model function of SAS was used to analyze serum inhibin A results within day of sample collection, using assay as a random variable. AMH levels were analyzed by one-way ANOVA (SAS), again within day of collection.

Generalized Linear Model Analysis:

To determine the predictive ability of both AMH and Inhibin A values on likelihood of breeding success, data were analyzed first by data before breeding
(pre-breeding samples) and day of breeding (breeding samples) by a Generalized Linear Model function of SAS (GENMOD procedure) to generate Wald Chi-square probability values with AMH concentration as the dependent variable, and inhibin concentration in pregnant cycles as the independent variable. Figure 1.

RESULTS

Levels of AMH within one week of breeding (pre-breeding sample, n=27; 17 non-pregnant, 10 pregnant), or on day of AI (n=17; 9 non-pregnant, 8 pregnant) were not different in females by pregnancy status (p’s > 0.3, 0.5; Figure 1). Similarly, serum levels of inhibin A were not significant different by pregnancy status when analyzed pre-breeding (p=0.5), however on the day of AI there was a weak tendency (p=0.19) for females who did not become pregnant to have lower levels of inhibin A (3.4 ±3.2 vs 5.6±3.3; Figure 3). We attempted to determine if we could use information from both Inhibin A and AMH concentrations in serum on either the pre-breeding sample or the day of breeding to predict pregnancy success. None of the parameters we tested were significant in this modeling (all p > 0.2; Figure 3).
Figure 1: Results of AMH pre-breeding and during breeding samples (x-axis) of cows that did get pregnant (Y) and those that didn’t get pregnant (N) following AMH concentrations (y-axis). No significant effects were observed due to large variations in AMH values by animal.
Figure 2: Results of Inhibin A pre-breeding and during breeding samples (x-axis) of cows that did get pregnant (Y) and those that didn’t get pregnant (N) following Inhibin A concentrations (y-axis). There may be a relationship between unsuccessful pregnancies and reduction in Inhibin A concentrations in serum.
Figure 3. Representative of the highest level of significance for Generalized Linear Model Analysis with $p = 0.23$. No slope to line, therefore the residual probability of pregnancy by inhibin A level (y axis) not related to AMH level (x-axis).
DISCUSSION

In this study we analyzed Anti-Mullerian hormone and Inhibin A as potential biomarkers of fertility in dairy cattle. These two hormones are produced during follicle growth at different stages of the follicle development, which is why bringing them together might have brought some predictable information towards fertility [28]. AMH is produced mainly during the beginning/early stages of follicle growth while Inhibin A is produced at high levels in the dominant pre-ovulatory follicle [28][29]. With Inhibin A being produced by the dominant follicle as it is transformed into the corpus luteum (CL), higher levels may indicate a better-quality follicle is present on the ovary capable of sustaining a pregnancy [29]

Results in this study suggest that AMH and Inhibin A serum levels are not likely to predict fertility when analyzed individually. When combining our data from both hormones to see if there was potential for a better predictive result, there again was no significance for predictive ability in dairy cattle.

As shown in Figure 1, in AMH we found no significant effects and no overall patterns due to large variation in our data by animal (Pre-breeding P>0.3; Breeding P>0.5). The most significant finding of the study was shown in Figure 2 where Inhibin A resulted in having slightly better P-values (Pre-breeding P>0.5; Breeding P>0.18) indicating that there may be a correlation with unsuccessful pregnancies and reduction in Inhibin A concentrations in serum. Although Inhibin A had a better result, we aren’t certain that this relationship is true for dairy cattle because the P-value was still higher than 0.05 - indicating we still have a high likelihood this may indicate results from random noise. A greater sample size is needed for both hormones to result in greater confidence of their predictive ability in fertility.

Combining data from both hormones resulted in our analyses having no slope on the graph. This showed even the residual analyses generated by inhibin data and pregnancy status contrasted with AMH levels had a high probability of no actual correlation for levels of both hormones to predict pregnancy status (Figure 3).

Measuring AMH allowed for a reflection of follicular growth activity in each individual cow. AMH concentrations are reflective of the number of follicles produced with an estimate of 25% of cows having a low number of follicle count associated with a low AMH level [22]. A previous study by Jimenez-Krassel tested that heifers with the lowest AMH concentrations are removed from a herd at a
greater rate when compared to higher AMH producing cows [22]. It was found that a single determination of AMH concentrations in dairy heifers can be a method to diagnose/predict longevity of fertility making this hormone useful to determine overall fertility status but not fertility of an individual cycle [22].

Results in our present study supported this previous study during the breeding concentrations of AMH shown in Figure 1 that showed a higher concentration of AMH may increase chances for a successful pregnancy. However, upon statistical analysis we obtained a high p-value (>0.3), which leads to low confidence for an industry-wide dairy recommendation to use as a predictor of cycle fertility. Originally the study was to have a sample size of 30 cows, but due limitations an inconsistent sample size of 27 was meant some had both before and during breeding serum samples taken, while some only before or only during samples taken.

Inhibin’s are glycoprotein hormones high in follicular fluid with two different forms existing (Inhibin A and Inhibin B), that affect FSH production to regulate follicular development [24]. Inhibin A and B fluctuate with opposite concentration levels during the follicular and luteal phases [25]. Inhibin A concentrations increase as the dominant follicle begins to ovulate and increase again to be high during the luteal phase, while Inhibin B is produced at high levels in smaller growing follicles like AMH [25]. Combining Inhibin B and AMH for a potential predictor of fertility was irrelevant for this current study because they both are produced at the early stages of follicle development.

Inhibin A functions as a negative feedback regulator for follicle stimulating hormone (FSH) secretion within the estrous cycle and postpartum period [26]. A lasting dominant follicle holds Inhibin A production at longer periods compared to an emerging dominant follicle from the estrous cycle [26]. In a previous study, Inhibin A rose to maximal levels during ovulation in bovine [20]. Results from our present study complemented this past result addressing that there tended to be higher concentration of Inhibin A in the breeding samples with successful pregnancies compared to those that did not get pregnant that cycle. But, again with the marginal P-value (P=0.18) there is no guarantee that all dairy cows will provide similar results. Additional animals are needed, especially those with samples collected at both time points of pre-breeding and again at breeding, to justify further use of Inhibin A to predict pregnancy in dairy cattle.

Completion of this study was affected by several factors. Heat stress for instance, was an uncontrollable limitation that is known to affect reproductive processes in dairy cattle [27]. The months of June and July there were still samples
being taken for the study and during that time there were extremely hot temperatures with a high of ~110°F with heat waves spiking throughout the weeks. Follicles and oocytes are highly sensitive reproductive components to elevated temperatures [27], and dominant follicles exposed to elevated temperatures may not form corpora lutea properly. Rising temperatures cause internal dairy cattle body temperatures to rise, especially to those that are lactating, and of our 27 cows used majority were lactating cows [27]. The reason heat affects them is due to the milk synthesis along with secretion working in combination to increase a cow’s metabolic heat produced, and increase harmful cellular by-products associated with metabolic stress [27].

Another limiting factor deviating our results was the lack of staff. At the time of breeding there was only one trained artificial insemination worker. Majority of trained dairy staff had graduated or didn’t work at the dairy anymore. This made it difficult to breed in a timelier manner as well as heat detect over the summer in an accurate method. With natural heat, and use of exogenous hormones, keeping track of all cattle was a lot of work to keep track of. Overall, the staff did a great job in managing as best as possible given the labor shortages.

Other limits kept in mind that could have influenced results were breed type, mixed age groups of cattle, and assay limitations. AMH concentrations in cattle have an indirect correlation to the total number of healthy ovarian follicles produced [30]. There has also been a correlation showing that AMH concentrations increase in the first months of a cow’s life, and then decrease before puberty which varies depending on breed as well [30]. Beef cattle are known to have higher AMH levels than dairy breeds [30]. In the current study some of our cows may have had different levels of inhibin A and AMH due to the number of breeding’s they’ve previously had; some may have been repeat breeders. I would have expected the younger first-time heifers to have had better results compared to the older cows. An amalgamation of all variables could have misdirected or unknowingly influenced our data on which cows had successful pregnancies regardless of hormone levels.

Future studies will be necessary to determine if Inhibin A can be a suitable biomarker for fertility in dairy cattle along with AMH. A future study considered after completion of this one was taking these hormones into an in vitro setting. This will involve collecting oocytes and allowing them to develop in an in vitro process where we would measure both hormones as the oocyte develops into an embryo. Measuring levels of both hormones and gathering data of each embryo
may allow us to see a possible correlation of which embryos were most successful at what level of each hormone’s concentration.

CONCLUSION

Diagnosing ovarian dysfunction in cattle can be time-consuming leading many producers to cull otherwise healthy animals with poor reproductive performance as an immediate option. There have been many studies done aiming to understand how fertility can be further understood with the use of reproductive hormones. Nonetheless, our results do support the conclusion that AMH and Inhibin A are possible biomarkers of fertility in dairy cattle, but it is not reasonable yet to say that AMH and Inhibin A combined make a good predictor of fertility for all dairy cattle. Future studies will be necessary to determine if Inhibin A can be a suitable biomarker for fertility in dairy cattle along with AMH.

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CONFLICT OF INTEREST

None declared
REFERENCES


