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Use of anti-mullerian hormone to select for fertility in beef heifers

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Abstract

A study was conducted to determine whether concentration of serum Anti-Mullerian Hormone (AMH) at weaning and/or breeding could predict subsequent fertility in beef heifers. Frequency distribution was used to assign serum AMH concentration measured at weaning, breeding, and the change from weaning to breeding into quartiles. Comparison of heifers based on serum AMH quartiles at weaning failed ($P \geq 0.35$) to detect any effect of AMH on subsequent heifer cyclicity at breeding, estrous response after synchronization, artificial insemination (AI) pregnancy rate, overall breeding season pregnancy rate, or estimated estrous cycle of the breeding season when conception occurred. Based on AMH concentration at breeding, heifers in the lowest quartile (Q1) had a lower ($P = 0.02$) AI pregnancy rate than heifers in other quartiles, and conceived at a later estrous cycle ($P = 0.03$) in the breeding season. Comparison of heifers based on the difference between AMH concentrations at breeding versus weaning revealed that none of the heifers in the lowest quartile (Q1) became pregnant after AI, compared with 80% in the highest quartile (Q4; $P < 0.001$). Heifers in the lowest quartile also conceived at a later estrous cycle in the breeding season than heifers in the other quartiles ($P = 0.01$). Results indicate that either AMH concentration at breeding or the change in AMH from weaning to breeding can identify beef heifers more likely to conceive to AI and to conceive early in the breeding season.

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Meet the Student-Author

Hannah Newberry

I was raised in Harrison, Arkansas where I graduated as valedictorian from Harrison High School in 2012. I chose to continue my education at the University of Arkansas, graduating summa cum laude in May 2016 with a major in Animal Science with the Pre-Veterinary concentration. Throughout my undergraduate career, I was a member of the Pre-Vet club for three years which helped me gain useful insight in order to apply for vet school in the summer of 2015. I have also been working part time as a veterinary assistant at the Harrison Animal Clinic for the past two years. In April 2016 I was accepted to the Oklahoma State University College of Veterinary Medicine Class of 2020. My future plans include pursuing a career in both large and small animal medicine.

I would like to thank my mentor Rick Rorie for all of his guidance and making this research project possible. I truly appreciate all of the time and effort he put into helping me. In addition, I would also like to thank my other two committee members, Beth Kegley and Charles Rosenkrans for their support for my thesis project and many other aspects of my undergraduate experience. Recognition should also be given to Toby Lester, who completed the ultrasonography and blood sampling, and Mohan Acharya and Chris Hansen who assisted with the anti-Mullerian Hormone assays for the project.

Introduction

The number of follicles present in the ovaries of heifers at birth range from 10,000 to 350,000 (Erickson, 1966). Heifers with low follicle counts also have smaller ovaries and fewer morphologically healthy follicles and oocytes, suggesting a link between follicle number and fertility (Ireland et al., 2008). Anti-Mullerian Hormone (AMH) is produced by granulosa cells of various size follicles (up to a size of 4 to 5 mm diameter), and reflects the total number of healthy follicles within the ovaries (Visser et al., 2006). Therefore, the measurement of AMH in circulation might be used as an indicator of fertility.

Anti-Mullerian Hormone is detectable as early as 36 weeks gestation in the ovarian follicles of developing heifer calves (Rajpert-De Meyts et al., 1999). A single measure of AMH in the circulation of breeding age heifers has been used to identify heifers with greater reproductive potential (Ireland et al., 2011). The question arises as to how early in development AMH can be measured as an indicator of fertility. Identification of heifers with low or high fertility at birth or weaning would be advantageous to producers for making management decisions. Therefore, the objective of the present study was to examine the relationship between serum AMH concentration at weaning versus breeding, and to determine if either or both measures could predict subsequent fertility of beef heifers.

Materials and Methods

Animal Management

The study utilized 71 beef heifers located at the University of Arkansas System Division of Agriculture's Beef Research Unit near Savoy, Arkansas. Prior to the study, all proposed animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC protocol # 15041). At weaning (~7 months of age), a 10-mL blood sample was collected in serum separator tubes, labeled, and the serum was frozen (-20 °C) until analysis for Anti-Mullerian Hormone (AMH). The heifers were then developed and maintained on pasture, with access to free-choice mineral, and provided corn gluten feed to meet energy requirements as needed. At breeding age (~14 months of age), a second 10-mL blood sample was collected in serum separator tubes, serum recovered and frozen.

Approximately 30 days before the start of the breeding season, transrectal ultrasonography (IBEX Pro with a L6.1 linear array transducer; E.I. Medical Imaging, Loveland, Colo.) was performed to determine the reproductive tract score (Anderson et al., 1991) of each heifer. At the time of reproductive tract scoring, a scan through the left and right ovary of each heifer was video recorded in order to accurately determine ovary size, the presence or absence of a corpus luteum, and the number and size of the larg-

est follicles present. Based on ovary size and structures present, heifers were categorized as cyclic or non-cyclic.

Estrous Synchronization and Breeding

At the start of the breeding season all heifers received a single 25-mg intramuscular (i.m.) injection of prostaglandin F2alpha (Lutalyse; Zoetis, Florham Park, N.J.) and an estrous detection patch (Estrotect; Rockway Inc., Spring Valley, Wis.). Heifers were observed 3 or more times daily for onset of estrus, and inseminated approximately 12 hours after detected estrus. Heifers not detected in estrus received a second Lutalyse i.m. injection 7 days after the initial treatment. Estrus detection and insemination continued for 4 days as previously described. Ten days later, the heifers were exposed to fertile bulls for a 45-day breeding season. Bulls were rotated through breeding groups half way through the breeding season. At 50 to 60 days after insemination, transrectal ultrasonography was used to identify pregnant heifers and to confirm conception date, based on fetal crown-to-rump length. At 60 days after bull removal, transrectal ultrasonography was used again to determine pregnancy in heifers conceiving during the breeding season and confirmed a continuing pregnancy in heifers previously identified as pregnant. Based on fetal size at ultrasonography, the estrous cycle after initiation of breeding when conception occurred was estimated. For comparison, artificially inseminated (AI) pregnancies were considered cycle 0, and pregnancies initiated during the first, second or third 21-day intervals of the breeding season were classified as cycles 1, 2 and 3, respectively.

Anti-Mullerian Hormone Assay

Serum samples were analyzed for AMH, using bovine AMH ELISA kits (Ansh Labs, Texas), and following procedures as outlined by the kit. Each assay plate contained a standard curve in duplicate, ranging from 0 to 2.4 ng/mL AMH. Two kits were utilized: one for the serum samples collected at breeding and the other at weaning. The ELISA kit was a 3-step sandwich type immunoassay using 96-well plates, with each well coated with biotinylated AMH antibody. Standards, high and low controls, and unknowns (50 µl) were added to appropriate wells, along with 50 µl of assay buffer. Each assay plate was then incubated 2 hours on an orbital plate shaker (Titer Plate Shaker, Lab-Line Instruments, Melrose, Ill.) at room temperature. Plates were then washed 5 times, using an automated plate washer (ELP-40 Microplate Strip Washer, Bio-Tek Instruments, Winooski, Vt.).

An AMH antibody-biotin conjugate (100 µl) was added to each well, followed by another incubation on the plate shaker for 1 hour. After washing 5× again, 100 µl of streptavidin-enzyme conjugate was added to each well, followed by incubation on the plate shaker for 30 minutes. Following another 5× plate wash, 100 µl of Tetramethylbenzidine (TMB) chromogen was added to each well and the plates placed back on the plate shaker. Visual color change was monitored and after 12 minutes, the plate was removed and 100 µl of stopping solution was added to each well to prevent further color change. Within 15 minutes of addition of stopping solution, the plates were read (0.1 second/well) for absorbance at 450 nm, using a Perkin-Elmer (Waltham, Mass.) Victor V, Model 1420 Multi-label Counter. Absorbance readings for 'blank" wells were subtracted from all other well readings to correct for plate optical density.

Statistical Analysis

All data analysis was performed using JMP Pro 12.0 statistical software (SAS Institute, Inc., Cary, N.C.). Regression analysis (bivariate fit) was used to determine the relationship between absorbance readings and standard concentrations of AMH. The resulting regression equations were used to calculate AMH concentration in each unknown sample within the appropriate assay plate. Frequency distribution was also used to assign AMH concentration measured in serum samples at weaning and breeding to quartiles. In addition, quartiles were established for the difference or change in AMH from weaning to breeding (breeding-weaning AMH). Comparisons were then made for heifers in each quartile and the percentage of heifers cyclic at synchronization, expressing estrus after synchronization, conceiving after artificial insemination, pregnant at the end of the breeding season, and the estimated cycle after the initiation of breeding that conception occurred.

Results and Discussion

The heifers weighed an average of 240.6 ± 2.5 kg at weaning and 358.6 ± 3.7 kg at the start of the breeding season. Transrectal ultrasonography determined that 39/71 (55.0%) of the heifers were cyclic before the start of breeding. After 2 (7 days apart) injections of prostaglandin F2alpha to induce estrus, 48/71 (67.6%) of heifers were detected in estrus and inseminated. Twenty-two of forty-eight (45.8%) of the heifers conceived after artificial insemination. At ultrasonography ~60 days after the breeding season 62/71 (87.3%) of the heifers were confirmed to be pregnant.

The inter-assay variation among duplicate samples in the AMH assay performed for serum samples collected at weaning was 2.7%. The regression equation (R^2 = 0.998) used to determine AMH concentration in serum collected at weaning was: AMH ng/mL = -0.034853 + 0.5789461*absorbance. At weaning, serum AMH

ranged from 0.04 to 0.99 ng/mL, with a mean of 0.30 ng/mL. The inter-assay variation among duplicate samples in the AMH assay performed for serum samples collected at breeding was 3%. The regression equation (R^2 = 0.995) used to determine AMH concentration in serum collected at breeding was: AMH ng/mL = -0.080924 + 0.577811*absorbance. At breeding, serum AMH ranged from 0.04 to 1.73 ng/mL, with a mean of 0.56 ng/mL.

When heifers were compared by quartiles, based on serum AMH at weaning, AMH hormone concentration at that time had no effect ($P \geq 0.35$) on subsequent heifer cyclicity at breeding, response to synchronization, AI pregnancy rate, overall pregnancy rate, or mean cycle of the breeding season when conception occurred (Table 1). Failure to detect an effect of AMH concentration at weaning on subsequent fertility in beef heifers is in contrast with a study conducted with sheep. Lahoz et al. (2012) measured plasma AMH in 76 ewes at 3.6 months of age. The ewes were mated at 10 months of age, with those failing to conceive being mated again 4 months later. Results of that study indicated that fertility of ewes at first mating positively correlated with circulating AMH concentration at 3.6 months of age. The study concluded that a single AMH measurement performed on ewes at an early age was useful for selection of ewes with higher fertility potential at first mating.

Based on AMH concentration at breeding, heifers in the lowest quartile (Q1) had a lower ($P = 0.02$) AI pregnancy rate than heifers in other quartiles and conceived at a later cycle ($P = 0.03$) in the breeding season (Table 2). Studies have shown that heifers conceiving early in their first breeding season will continue to conceive early in subsequent breeding seasons, wean heavier calves, and be more productive throughout their life (Bellows and Staigmiller, 1994). Recently, Jimenez-Krassel et al. (2015) measured AMH on 11 to 15 month old Holstein heifers before first breeding, and then followed their reproductive performance and productivity through two lactations. Compared to heifers in higher AMH quartiles, heifers in the lowest AMH quartile on average had a productive herd life that was 196 days shorter, the lowest level of first lactation milk production, the lowest percentage for cows pregnant across all lactations, and the highest culling rate for poor reproduction.

Plasma AMH concentration in bovine females has been reported to remain relatively stable throughout the first year of life (Rota et al., 2002). In the current study, mean AMH in serum increased from 0.30 at weaning to

Table 1. Effect of serum anti-Mullerian hormone (AMH) concentration at weaning on cyclicity and pregnancy rate in beef heifers.

	Anti-Mullerian hormone quartile				
Item	**1**	**2**	**3**	**4**	***P*-value**
AMH range (ng/ml)	0.04 - 0.15	0.17- 0.24	0.25 - 0.38	0.40 - 0.99	
Cyclic at breeding (%)	8/17 (47.1)	11/18 (61.1)	8/16 (50.0)	9/17 (52.9)	0.855
Synchronized estrus (%)	10/17 (58.8)	14/18 (77.8)	10/18 (55.6)	13/17 (76.5)	0.354
AI pregnancy rate (%)	4/10 (40.0)	8/14 (57.1)	5/10 (50.0)	5/13 (38.5)	0.754
Overall pregnancy rate (%)	15/17 (88.2)	16/18 (88.9)	17/18 (94.4)	13/17 (76.5)	0.449
Mean conception cycle	1.33	0.81	1.24	1.00	0.523

AI = artificial insemination.

Table 2. Effect of serum anti-Mullerian hormone (AMH) concentration at breeding on cyclicity and pregnancy rate in beef heifers.

	Anti-Mullerian hormone quartile				
Item	**1**	**2**	**3**	**4**	***P*-value**
AMH range (ng/ml)	0.04 - 0.23	0.27 - 0.45	0.50 - 0.77	0.80 - 1.73	
Cyclic at breeding (%)	7/17 (41.2)	11/18 (61.1)	12/17 (70.6)	7/17 (41.2)	0.206
Synchronized estrus (%)	10/17 (58.8)	13/18 (72.2)	14/18 (77.8)	11/18 (61.1)	0.572
AI preg. rate (%)	1/10 (10.0)^a	7/13 (53.9)^b	6/14 (42.9)^b	8/11 (72.7)^b	0.021
Overall preg. rate (%)	14/17 (82.4)	16/18 (88.9)	16/18 (88.9)	16/18 (88.9)	0.919
Mean conception cycle	1.79^a	1.0^b	0.75^b	0.94^b	0.034

^{a,b} Within rows, numbers with different superscripts are significantly different ($P < 0.05$).
AI = artificial insemination.

0.56 ng/mL at breeding. It was also noted that the serum AMH concentration of some individual heifers either did not increase or actually decreased during this time. Therefore, heifers were assigned to quartiles based on the difference between AMH concentration at breeding and weaning (Table 3). None of the heifers became pregnant after AI in the lowest quartile (Q1), compared with 80% in the highest quartile (Q4; $P < 0.001$). Heifers in the lowest quartile also conceived at a later cycle in the breeding season than heifers in the other quartiles ($P = 0.01$).

In the study previously mentioned that was conducted with dairy heifers (Jimenez-Krassel et al., 2015) it was hypothesized that AMH concentration had a positive correlation with high antral follicle counts, fertility, and ovary function. The study confirmed that a single blood sample for AMH from breeding age dairy heifers could be used to select replacements and predict long-term reproductive performance of dairy heifers. Often reproduction is negatively correlated with other desirable traits. However, the results of Jimenez-Krassel et al. (2015) showed that AMH could be used to identify more fertile heifers without compromising milk production.

A study utilizing 1237 multiparous dairy cows of three different breeds determined if circulating AMH had a direct relationship with fertility during a planned 100-day breeding season (Ribeiro et al., 2014). The cows were synchronized, and either placed in timed insemination protocol or inseminated at estrus. Serum samples were collected on day eight of the estrous cycle for measurement of both AMH and progesterone. Concentrations of AMH were found to vary among the breeds of cows and those at different stages of lactation. Although no relationship was found between AMH levels for dairy cows enrolled in timed insemination, a positive correlation was found between AMH and pregnancy rates with dairy cows bred after detected estrus. In addition, Ribeiro et al. (2014) found pregnancy loss to be greater in cattle with lower AMH.

A study in goats reported that AMH could be used as a predictor of in vivo embryo production (Monniaux et al., 2011). Plasma AMH was measured in goats before follicle-stimulating hormone (FSH) treatments were given to stimulate follicular growth at the beginning of the breeding season, at the end of the breeding season, and during the anestrus period. High AMH was positively correlated with higher numbers of corpora lutea and embryo recovery. The study concluded that AMH could help predict the ability of goats to respond to the superovulatory treatment, as well as whether they will produce high numbers of transferable embryos. It was noted that the goats' plasma AMH concentrations gradually decreased after each embryo collection.

Results of the current study and others concur that AMH can be used as a predictor of fertility in replacement animals. This study concluded that AMH concentration at breeding and/or the change in AMH from weaning to breeding showed a positive correlation between AMH and fertility. Lahoz et al. (2012) measured AMH in sheep during the prepubertal period and found a correlation between AMH and fertility later in life. In goats, a positive correlation was measured between AMH and in vivo embryo production after superovulation. In both dairy heifers and mature cows, circulating AMH was shown to be positively correlated with fertility (Jimenez-Krassel et al., 2015; Ribeiro et al., 2014).

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Table 3. Effect of change in serum anti-Mullerian hormone (AMH) concentration from weaning to breeding on cyclicity and pregnancy rate in beef heifers.

	Anti-Mullerian hormone quartile				
Item	**1**	**2**	**3**	**4**	***P*-value**
AMH range (ng/ml)	-0.48 - 0.04	0.05 - 0.16	0.17 - 0.44	0.48 - 1.32	
Cyclic at breeding (%)	7/17 (41.2)	12/18 (66.7)	10/17 (58.8)	7/16 (43.8)	0.374
Synchronized estrus (%)	10/17 (58.8)	14/18 (77.8)	13/18 (72.2)	10/17 (58.8)	0.525
AI preg. rate (%)	0/10 (0.0)[a]	7/14 (50.0)[b]	7/13 (53.9)[b]	8/10 (80.0)[b]	>0.001
Overall preg. rate (%)	13/17 (76.5)	16/18 (88.9)	17/18 (94.4)	15/17 (88.2)	0.464
Mean conception cycle	1.92[a]	0.93[b]	0.88[b]	0.80[b]	0.014

[a,b] Within rows, numbers with different superscripts are significantly different ($P < 0.05$).
AI = artificial insemination.

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