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Potential Importance of Circulating anti-Müllerian Hormone as a Predictor of Superovulatory Response in Dairy Holstein Cows

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Abstract

Plasma anti-Müllerian hormone concentration (AMH) in cows is a useful endocrine marker for ovarian response to gonadotropin superstimulation. Here, we investigated the possible relationship between AMH concentration measured in plasma and superovulatory response of embryo donor dairy Holstein cow aiming to predict their response in earlier period before beginning of superovulatory treatment protocol. So, for this reason, 33 cows were undergo superovulatory gonadotropin treatment and blood samples were collected at three phases (P_0 randomly before treatment, P_E on day of estrus, P_F on day of flushing for embryos collection). This study revealed that AMH concentration were significantly increased in the high responded cows in all 3 phases of study than the low responded ones. Also, the study reported 3 optimal cut-off points 57.6, 192.1 and 114.2pg/ml in the P_0, P_E and P_F phases respectively which could be used for early prediction of cow superovulatory responses. In conclusion, circulating AMH concentration was strongly associated with superovulation response and its evaluation could be used to identify embryo donor cows with greater response to superovulation programs. Also, the optimal cut-off point 57.6pg/ml could be used randomly to select the best donors

(Key wards: Superovulation, anti-Müllerian Hormone (AMH), dairy cows)

Introduction

Recent advances in bovine biotechnology, such as commercially available genomic testing, have allowed for the identification of animals with superior genetics. Genetic selection and reproductive efficiency are key factors for the success of the dairy and beef industries. In cattle, Multiple Ovulation and Embryo Transfer (MOET) programs have become a large international business. More accurate identification of cows with greater embryo production potentials could allow for more efficient production of in vitro and in vivo bovine embryos for cows with superior genetics (**Rico et al., 2009**). However, cost efficient propagation of these superior genetics has been hampered by high variability between animals in response to embryo production techniques such as superovulation (**Souza et al., 2015).** Despite improvements in superovulatory treatments, ovarian responsiveness to gonadotropins remains highly variable between individuals and difficult to predict. It is well established that the major source of variability is the status of ovarian follicles at the time of initiation of FSH treatment (**Rico et al., 2009**).

Presently, some clinical studies thrown its light on anti-Müllerian Hormone (AMH) describing it as the best endocrine marker of ovarian follicular reserve, largely replacing other serum markers such as inhibin B, estradiol, basal FSH and LH (**Fanchin et al.,2003**; **Broekmans et al., 2006**; **Toner and Seifer**, **2013**). Anti-Müllerian Hormone is also the best predictive marker of the ovarian response to stimulatory treatment as defined by the number of oocytes retrieved In Assisted Reproductive Technology (ART) (**El-Gindy et al., 2008**). Moreover, information is also accumulating in cattle proving that measurement of circulating AMH concentrations may be the most reliable method for predicting not only fertility potential and reproductive longevity **(Baruseli et al., 2015; Jimenez-Krassel et al., 2015; Manal et al.,**

2016) but also the relative number of morphologically healthy follicles and oocytes in ovaries (**Ireland et al., 2008; Ireland et al., 2011**; **Monniaux et al., 2013; Batista et al., 2014**). Also, during the bovine estrus cycle two to four sequential waves of terminal follicular growth occur, each producing a dominant follicle capable of ovulating, if luteal regression occurs **(Fortune et al., 2001),** and variations in concentrations of AMH during emergence and regression of follicular waves remain to be established **(Rico et al., 2011)**

Anti-Müllerian Hormone (also known as Müllerian Inhibiting Substance, MIS) is a glycoprotein of 140-KDa that is member of transforming growth factor-β superfamily (TFG-β) of growth and differentiation factors, knowing as a gonadal hormone expressed only in gonads (**Pepinskey et al., 1988**). The sexually dimorphic regulation of AMH expression is a fruitful area of inquiry since it is early marker in mammals for the genetic switch that occurs when a bipotential gonads is instructed to differentiate into a testis in response to the testis-determining factor, SRY (Sex – determining Region of the Y-chromosome) (**Swain and Lovell,1999; Capel,2000**). Müllerian Inhibiting Substance (MIS) has long been known for its signature developmental effect of causing regression of Müllerian duct, the anlagen of fallopian tubes, uteri, cervix and upper vagina, a requirement of normal male reproductive tract development. Whereas,**T** ,another hormone produced by fetal testis , is required for Wallfian duct differentiation into male internal reproductive tract structures (**Teixeira and Donahoe, 1996**).

In the ovaries, AMH expression is restricted to a single cell type that is granulosa cells of growing follicles as recorded by previous studies in many species (in cows**, Vigier et al., 1984**; **Monniaux et al., 2008**, in sheep, **Bezard et al., 1987**, in human, **Rajpert et al., 1999**, **Weenen et al., 2004**). Anti-Müllerian Hormone is of key importance as it inhibits the recruitment of primordial follicles into the pool of growing follicles, and it decreases the responsiveness of growing follicles to Follicular Stimulating Hormone (FSH) (**Durlinger et al., 2002**). This pattern of expression makes AMH a reliable endocrine marker of the population of small antral gonadotropin responses follicles in the cows (**Rico et al., 2009**).

Thus, the objective of this study was to investigate whether circulating AMH concentrations could be used as a predictor for superovulation response by a single measurement before beginning of superovulatory treatment in dairy Holstein cows. In addition, to detect an optimal cut-off point which could be used to select the best donors.

Materials and Methods

Animal Housing and Diets:

Thirty-three multiparous dairy Holstein cows (3 to 7 years) old and their body weight ranged from 450 to 800 Kg.were used in this experiment which was conducted at a private farm at Alex-Cairo desert road. The experiment was done from November 2015 to January 2016. Experimental animals were assigned in an open yard and were provided feed and water *ad libtum*. Nutrient concentration met nutrient requirements of dairy cattle according to **NRC (2001).** They were fed concentrated ration (corn, soya bean meal and premix) in addition to afla afla and corn silage of high quality. The amount of ration was calculated according to the amount of milk production.

Experimental Design and Superovulation Protocol:

This experiment aimed to establish the relationship existing between circulating AMH concentration in plasma on one hand and ovulatory responses to a superovulatory treatment on the other hand. Table (1): Superovulation protocol **(Rivera et al., 2011)**.

Day of treatment	Time	Treatment
Three days before treatment	8 am	CIDR+GnRH
Day 1	8 am 8 pm	80 mg FSH (4ml) Same dose
Day 2	8 am 8 pm	60 mg FSH (3ml) Same dose
Day 3	8 am 8 pm	40 mg FSH (2ml) Same dose
Day 4	8 am 8 pm	20 mg FSH (1ml) + PGF2α Same dose+ CIDR remove
Day 6	8 pm	GnRH
Day 7	8 am 8 pm	Insemination by 2 straws Insemination by 2 straws
Day 8	8 am	Insemination by 2 straws
Day 15 (day of flushing)	8 am	Embryo collection

- Intravaginal progesterone (P4) implants (Eazi-Breed CIDR; containingn1.38g of P4, Pfizer Animal Health, New York, NY).
- GnRH (Buserlin acetate, 1.0 µg/dose, Receptal; Intervet, International GmbH, Germany).
- FSH (400mg, Folltropin-V; Bionich Life Sciences, Belleville, ON, Canada).
- PGF2α (Estrumate, 250µg of cloprostenol sodium/dose; Shering-Plough Animal Health, Union,NJ).

Cows were inseminated three times at 12hrs. interval by 2 straws of frozen semen (15x 10^6 sperm/straw) from Holstein sire with high genetic merit, proven outstanding field fertility (sire conception rate scores ≥2).

CL Counting and Embryos Collection:

Donor was contained in a crush, usually with head-tail restraint. Then a well-trained vet examined both ovaries for determining the total number of CL that may be corresponding to the total number of embryos may be collected. We used an epidural anesthetic for relaxation of the rectum and local effect on the tract to aid manipulation. The collection catheter is passed through the cervix and located in place usually in each of the uterine horns in succession, by manipulation with a hand insertion in the rectum (similar to manipulation of the cervix during AI). When the catheter is correctly located, the cuff is inflated and flushed medium passed through to collect the embryos. Then they were located and retrieved from the flushed fluid using a microscope, assessed for quality on appearance and stage of development, and then either prepared for transfer or frozen for storage.

Blood Sampling:

By jugular vein puncture, blood samples from each cow were received in clean and sterile tubes containing EDTA at three different phases:

$\mathbf{P_0}$ representing the samples that were taken before superovulatory treatment (random).

$\mathbf{P_E}$ representing the samples that were taken after superovulatory treatment at time of estrus.

$\mathbf{P_F}$ representing the samples that were taken 7 days after estrus where counting of CL was done and flushing for embryos collection was taken place.

Collected blood samples were immediately kept in icebox and later centrifuged at 3000rpm/15 min. for plasma separation. Then samples were kept frozen at -20 untill AMH assay were done.

Plasma AMH Assay:

Bovine anti- Müllerian Hormone was assayed by ELISA technique using commercial diagnostic kit (**Nova** Tec, Immudiagnostica GmbH Waldstraβe 23 A6, D-63128 **Dietzenbach, Germany**). The assay had a sensitivity of 0.04pg/ml and coefficient of variation of 2.2٪. This assay had high sensitivity and excellent specificity for detection of bovine AMH. No significant cross-reactivity or interference between bovine AMH and analogues was observed.

Statistical Analyses:

Statistical analyses were performed using Sigma Plot version 13 (Systat Software Inc. San Jose, CA). The sensitivity and specificity of the test were calculated according to the formula that use the median values to determine the high (true positive) and low (true negative) responded cows to superovulation treatment in addition to false positive and negative responded ones.

Sensitivity = True positive /True positive + False negative.
Specificity = True negative/ True negative + False positive.

Optimal Cut-off points were calculated using the Receiver Operating Characteristics (ROC) curve analysis feature in Sigma Plot that indicate whether the cows can be classified into high or low responded cows to superovulatory stimulation **(Gardner and Greiner2006; Mercaldo et al.,2007).**

Results

As assessed by hormonal assay in the studied cows from the present experiment, high between- animal variations were observed for plasma anti-Müllerian hormone concentrations and the ovarian response. Fig 1 (A, B, C and D), there was a positive relationship between AMH measurements done on individual cows at different phases of the estrous cycle (P_O xP_E), (P_O X P_F) and (P_E X P_F). Most measurements were found to be close to the predicted line of unity between each of the assays. Additionally, the relationship between the AMH concentration in P_O phase and number of CL showed the same manner.

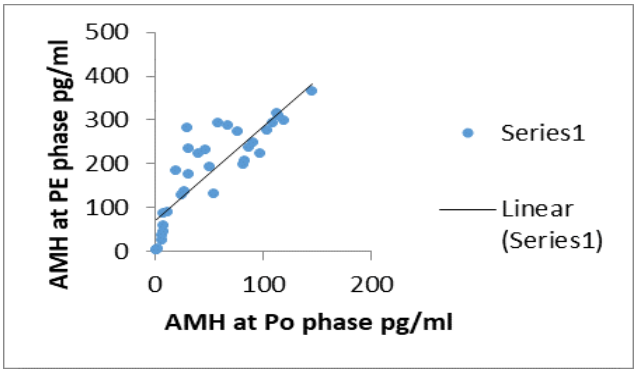


Figure 1 (A): showing measurement of AMH at P_o phase (random stage of the cycle) versus AMH in P_E phase

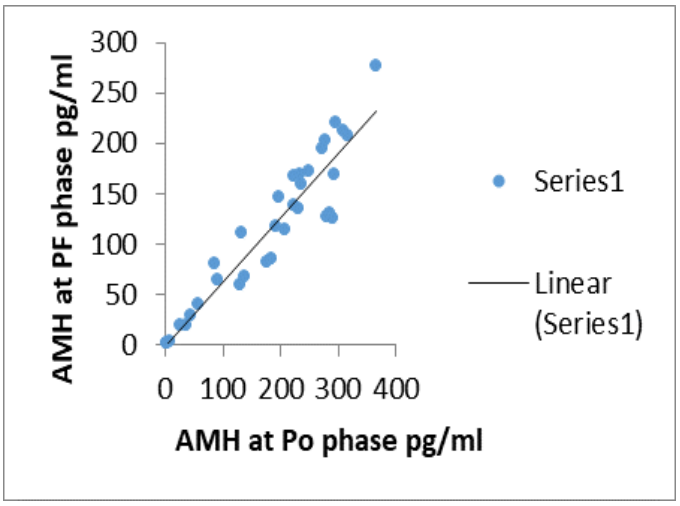


Figure 1 (B): showing measurement of AMH at P_o phase (random stage of the cycle) versus AMH in P_F phase

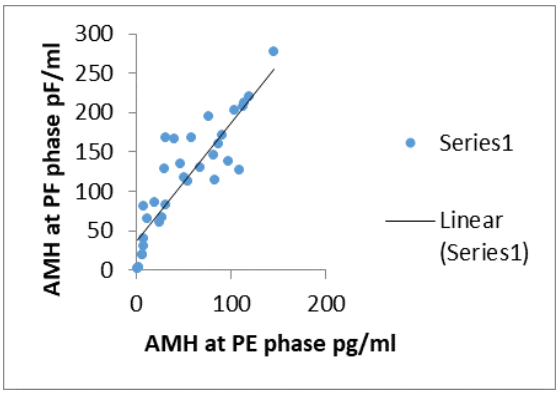


Figure 1 (C): showing measurement of AMH at $P_{E\ PPE}$ phase versus AMH in P_F phase

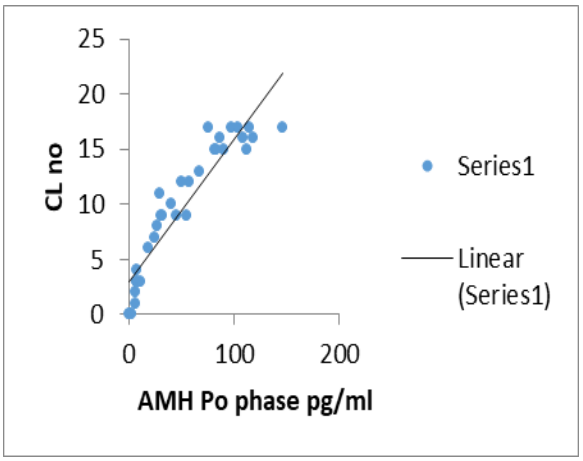


Figure 1 (D): showing measurement of AMH at P_o (random stage) and number of corpus luteum (CL) on the day of embryo collection

Statistical results will present the descriptive statistics, comparative analysis and ROC curve (Receiver Operating Characteristics) for AMH concentration in 3 phases of investigation (P_0, P_E and P_F) and CL responses in all cows under study. As reported in many studies, the descriptive statistics goals to show the elementary characteristics for our data, however the comparative analysis investigates whether there is statistical significance between the high and low response to gonadotropin stimulation with respect to P_0 , P_E and P_F where cows are classified into high response if CL number was greater than the median value (10 CL) and vice versa. In addition, ROC curve is obtained separately for P_0 , P_E and P_F in order to estimate the optimal cut-off points that indicate whether the cows can be classified into high or low response with respect to the number of CL.

Table (2): The Descriptive Measures for Plasma AMH Concentration (pg/ ml) and CL Number in Cows under Study

Phase	No.	Mean	Median	Std. Deviation	Min.	Max
P_0	33	53.1061	45.9000	42.40822	0.8	145.9
P_E	33	184.5691	207.1000	106.21177	1.4	365.5
P_F	33	116.7845	126.3000	72.08333	1.1	277.3
CL	33	9.8182	10.0000	5.94482	0.0	17.0

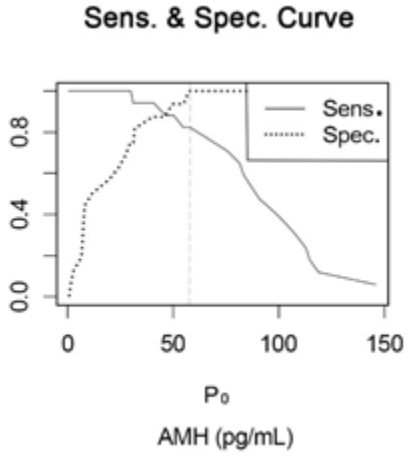
Table (2) presents the basic statistical measures for the cows in the 3 phases of the study beginning with the number of the teste

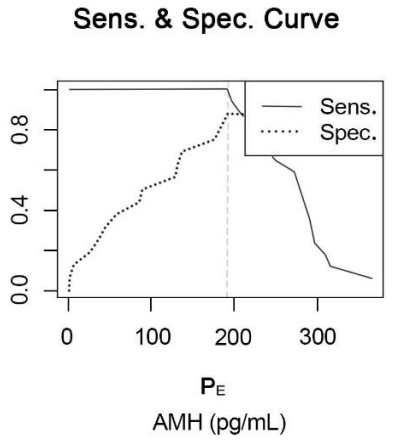
Table(3): Comparison between Concentrations of AMH (pg/ml) in High and Low Responded Cows for Gonadotropin Stimulation with Respect to The Number of CL (Mean ± S.E.)

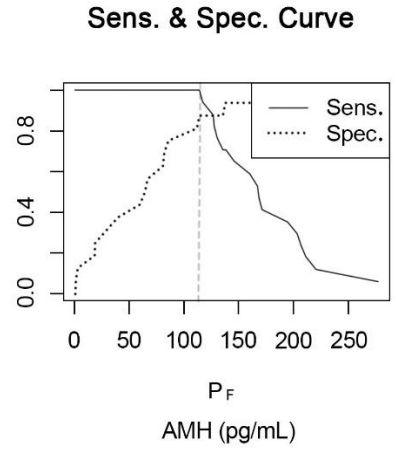
Phase	High Response N=17	Low Response N=16	P-value
P_0	86.24±30.61	17.89±16.41	<0.01
P_E	265.81±47.45	98.24±78.96	<0.01
P_F	169.62±44.57	60.63±49.37	<0.01

Table (3) shows that there was a significant difference (P<0.01) between the high and low response cows for gonadotropin stimulation in each period. In addition, all the averages of the low response cows less strictly than high ones.

Fig (2): Estimated optimal Cut-off points for cows in the 3 examined phases







According to Fig (2) ROC plots, the estimated optimal cut-off point for AMH concentration in three examined periods P_0, P_E and P_F were 57.6, 192.1 and 114.2 pg/ ml respectively. Which refers to that cows whose AMH concentrations were greater than that values, were classified as high responded for gonadotropin stimulation and vice versa

Discussion

A valid assessment of the hypothesis that AMH concentrations are predictive of superovulatory response necessitates a standardized, consistent superovulation protocol and a validated, repeatable AMH assay **(Souza et al., 2014).** For the superovulation protocol, in this study, we used the same total decreasing dose of FSH (400mg) for all cows standardize superstimulation. In addition, we tried to ensure that circulating **P4** concentrations were high by using **CIDR** present during superstimulation **(Rivera et al., 2011)**.

This study recorded high between- animal variations were observed for plasma anti-Müllerian hormone concentrations and the ovarian response (CL number) which was agreed with the study previously made by **Rico et al., (2009).**

The present study proved that there was a positive relationship among the AMH concentrations done on individual cows at different phases of estrous cycle. In agreement with **Souza et al. (2015)** AMH follows a specific profile during estrous cycle which occurs independently of the follicular waves of terminal follicular development. **Rico et al. (2011)** explained that profile consisted of a rapid decrease in AMH concentrations after estrus, reaching minimal values between day 4-8 of the cycle, followed by a slow increase until the next estrus.

Plasma AMH concentrations recorded a significant change between high versus low responded cows depending on the CL counting in day of flushing. Our observation of reduced superovulatory response in cows with low AMH concentration might be explained in part by reduced sensitivity to FSH in these cows; however, the most important explanation remains the reduced numbers of antral follicles that can respond to the exogenous FSH treatment **(Souza et al., 2015)**. Additionally, **Ireland et al., (2011)** illustrated that cows with low antral follicle counts have elevated circulating FSH concentrations, which could downregulate follicular FSH sensitivity to exogenous FSH stimulation. Moreover, **Scheetz et al. (2012)** demonstrated that bovine granulosa cells from cows with low antral follicle counts were less sensitive to FSH, in terms of FSH induction of in vitro estradiol production and

CYP19A1 mRNA expression, compared with granulosa cells from cows with high antral follicle counts. The authors explained that this may be due to expression of lower FSH-receptor mRNA in granulosa cells from cows with low versus high antral follicle counts.

This study also recorded a significant change in the plasma AMH concentrations between the 3 phases of the study (P_0, P_E, P_F). The first sample P_0 phase was taken randomly before gonadotropin stimulation which represented the normal plasma concentration of AMH. Therefore, it recorded the lowest AMH concentration when compared to other phases. While the highest AMH concentration was recorded in the P_E phase at the day of estrus after the cows subjected to superovulatory stimulation. Meanwhile, at the P_F phase the AMH concentration recorded a significant decrease when compared with the P_E phase but still significantly increased when compared with the P_0 random phase. **Vigier et al., 1984; Rico et al., 2009; Monniaux et al., 2013** stated that AMH expressed only from granulosa cells of preantral and small antral follicles. The significant increase of AMH concentration in P_E phase in the present study predicted an increase in the population of small antral and preantral follicles when compared with two other phases. Although the population of early antral follicles was not measured in this study.

Meanwhile, we recorded a significant decrease in AMH concentrations at the third phase **P_F** in comparison with the second phase P_E, although, its concentration is still significantly increased when compared with the first P_0 phase as the hormone is still expressed not from granulosa cells of growing preantral and small antral follicles but from cumulus cells and the outer layers of granulosa cells close to the theca. **Rico et al. (2011)** illustrated that these cells were shown to be preferred zones of high AMH expression in healthy antral follicles larger than 1.5 mm in diameter. Moreover, in atretic mouse follicles, AMH expression was strongly diminished except in the cumulus cells surrounding the oocyte as it can prevent cumulus cells apoptosis by maintaining a morphogenic paracrine gradient of BMP (Bone Morphogenic Protein) as stated by **Hussein et al. (2005).** From this point of view, we can predict that atretic follicles in high responded cows of this study were derived from healthy antral follicles and its size were larger than 1.5mm in diameter.

These measures indicated that the superovulatory method used in this protocol allowed us to precisely compare superovulation to plasma AMH concentration. The variation between individuals was substantial ranging from (0.8 to 365.5 pg/ml). We analyzed AMH at three different phases to determine the repeatability of the AMH value in individual cows. The high repeatability of AMH measurements across different phases of this study, makes AMH determinations particularly useful as a clinical diagnostic tool that provide sufficient information to establish a reasonable estimation of ovarian response to superovulatory treatment and ovarian reserve as previously shown in other studies **(Rico et al., 2009; Ireland et al., 2011; Monniaux et al., 2013; Souza et al., 2014; Hirayama et al., 2016).** The significant difference in AMH concentration between P_0 phase and its concentration in two other phases P_E and P_F add an important advantage for AMH testing to predict follicular reserve and superovulatory response to gonadotropin treatment. This result indicated that a single threshold for circulating AMH could be used to select donor cows at an early stage corresponded to the ovarian response to superovulation before the beginning of the superovulation protocol. For more confirmation, **Rico et al.** (**2009)** and **Monniaux et al. (2010)** indicated that AMH is also of interest in the context of multiple ovulation and embryo transfer (MOET) technology, which has significant role in genetic selection strategies in cattle, because plasma AMH concentrations of individual before gonadotropin treatment have

been found to be characteristic of each animal over a long-term period and predictive of the number of ovulations and embryos produced in response to ovarian gonadotropin treatments.

Thus, give AMH a great economic importance as using the same costs of housing, feeding, FSH, and labor, the expense per embryo would be less than half for embryo production from cows with high ovulatory response. So, AMH could be used strategically by embryo transfer practitioners to reliably select cows with high superovulatory capability and thus dramatically reduce costs of embryo production.

Determination of an optimal cut -off point for plasma AMH concentration allows us to select candidate embryo donor cows, even randomly, in an early stage before superovulatory stimulation. The current trail recorded three optimal cut-off points represented to the three investigated phases P_0, P_E, P_F which were 57.6, 192.1 and 114.2 pg/ml respectively. Previous studies on Holstein cows made by **Rico et al. (2012)** and **Souza et al. (2015)** recorded only one cut- off point by calculating the average of AMH concentrations from the three samples. **Rico et al. (2012)** recorded cut-off point 87 pg/ml using heparinized plasma and stated that AMH measured in frozen plasma maintained with EDTA was 1.5 times higher than if the same samples were maintained with heparin. **Souza et al. (2015)** recorded cut-off point 123.5 pg/ml using plasm maintained with EDTA, which nearly equal 130 pg/ml to that calculated by **Rico et al. (2012).** Surprisingly, our study recorded an optimal cut-off point 121.4 pg/ml which nearly similar value to that recorded by the two authors when we calculate the average concentration of AMH for the 3 phases; using ROC curve analysis of samples utilizing EDTA.

From these findings, we can take the first random cut- off point (P_0 sample) which equal 57.6 pg/ml to predict the response of cows to superovulatory treatment as early as possible.

Conclusion

In conclusion, circulating plasma AMH concentration was impressively accurate in predicting superovulatory responses in term of CL number and embryos produced by a cow in response to standardized superovulatory protocol. In addition, we can take the concentration of 57.6 pg/ml as a cut-off point to predict randomly the superovulatory response of candidate embryo donors in Holstein cows under the same circumstances. Although, further investigations in large scale using larger number on animals are needed to confirm this result.

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الملخص العربى

أهمية هرمون الانتى موليريان كدليل للتنبؤ بمدى استجابة المبايض للتنشيط في الابقار الهولشتين الحلابة

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*قسم بحوث بيولوجيا التكاثر ** قسم التلقيح الاصطناعى –معهد بحوث التناسليات الحيوانية بالهرم

يعتبر تركيز هرمون الانتي موليريان في بلازما دم الأبقار دليل جيد يمكن الاعتماد عليه للتنبؤ بمدى استجابة الابقار لتنشيط المبايض عند تطبيق البرنامج الهرموني المعد لذلك. وعليه تهدف هذه الدراسة لبحث تلك العلاقة هادفة الى انتخاب الابقار الأكثر استجابة والمنتجة لأكبر عدد من الاجنة وذلك في مرحلة مبكرة وقبل اعدادها ومعاملتها لبرنامج تنشيط المبايض الهرموني لما في ذلك من مردود اقتصادي كبير. ولهذا الهدف تم تطبيق برنامج تنشيط المبايض على 33 بقرة هولشتين حلابة تتراوح اعمارها بين 3-7 سنوات واوزانها من 450-800 كيلوجرام وتتم تغذيتها التغذية المناسبة للقطعان الحلابة. وقد تم سحب ثلاث عينات دم من كل حيوان ممثلة لثلاث مراحل لدورة الشبق. الأولى: عينة عشوائية وقبل معاملة الابقار هرمونيا لتنشيط المبايض. الثانية: في يوم ظهور علامات الشياع. والثالثة: في يوم جمع الاجنة وعد الاجسام الصفراء على المبايض. وقد اثبتت النتائج ان هناك زيادة معنوية في تركيز هرمون الانتى موليريان في بلازما دم الابقار الأكثر استجابة لهرمونات تنشيط المبايض وذلك خلال المراحل الثلاث للتجربة. كما تم من خلال الدراسة تحديد ثلاث نقاط فاصلة مميزة لكل مرحلة يمكن الاعتماد عليها لانتخاب الابقار الأعلى استجابة وهي على الترتيب 57.6, 192.1, 114.2 بيكو جرام لكل مليلتر. كما انه يمكن الاعتماد على تركيز الهرمون الأول 57.6 بيكو جرام لكل مليلتر كتركيز عشوائي لانتخاب الابقار المتوقع استجابتها العالية عند تطبيق برنامج تنشيط المبايض هرمونيا