RESEARCH ARTICLE

Mapping Ovarian Status: Antral Follicle Count and Plasma Anti-Müllerian Hormone Correlation as Determinants of GPG Protocol Efficacy in Anestrus and Cystic Cows

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Abstract

Evaluating ovarian functional reserve is essential for managing reproductive disorders in high-yielding dairy cattle. This study investigated the relationship between plasma Anti-Müllerian Hormone (AMH) concentrations and Antral Follicle Count (AFC) in postpartum crossbred cows affected by anestrus and cystic ovarian degeneration (COD) following a Gonadotropin Releasing Hormone-Prostaglandin-Gonadotropin (GPG) therapeutic protocol. Thirty postpartum crossbred cows (2^{nd} -4^{th} parity) were allocated into three groups (n=10 each): Group I (COD), Group II (postpartum anestrus), and Group III (normal cyclic control). Reproductive status was assessed using transrectal ultrasonography and per-rectal palpation. All cows received a standardized GPG protocol. Blood samples and ultrasonographic examinations were performed on Days 0 and 9 to evaluate plasma AMH concentrations and AFC. At Day 0, cyclic and COD cows exhibited significantly higher AFC (22.40±2.30 and 23.10±2.64, respectively) than anestrus cows (13.30±1.23; $p<0.01$). Plasma AMH concentrations followed a similar pattern, with lower values in anestrus cows (229.50±24.18 ng/L) than in cyclic (300.97±24.36 ng/L) and COD cows (301.97±27.30 ng/L). Following treatment, AFC increased significantly in all groups, with the greatest improvement observed in anestrus cows (28.70±1.71; $p<0.001$). Plasma AMH concentrations also increased significantly ($p<0.05$) in cyclic, anestrus, and COD cows. Strong positive correlations were observed between AFC and plasma AMH both before ($r=0.799$; $p<0.001$) and after treatment ($r=0.708$; $p<0.001$). Estrus induction and conception rates were highest in cyclic cows (90.0% and 66.7%), followed by COD (70.0% and 57.1%) and anestrus cows (60.0% and 50.0%). Although AMH showed a positive association with treatment response, its predictive value for conception was limited (AUC=0.665; $p>0.05$). Overall, plasma AMH reliably reflected AFC and follicular activity, while the GPG protocol effectively enhanced folliculogenesis in anestrus and COD-affected cows.

Keywords: Anestrus, Anti-Müllerian hormone, Antral follicle count, Crossbred cows, Cystic ovarian degeneration, GPG protocol.

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Introduction

Crossbred (*Bos indicus* x *Bos taurus*) cows are famous for their substantial milk production capacity, typically yielding between 4,500-5,500 kg over a lactation period of 257 to 350 days. The milk produced by these cows is characterized by high fat and protein content. Heifers reach an appropriate breeding age at approximately 13 months, by which time they generally weigh around 363 kg. It is customary for these cows to have their first calving at about 22 months of age, at which point they have attained approximately 80% of their mature body weight (NBAGR, 2017). Under semi-intensive system of management, reproductive disorders are the largest, where repeat breeding (22.4%), postpartum anestrus (19.6%) and fetal membrane retention (14.7%) are associated with nutritional imbalances (e.g., negative energy balance), inadequate housing and infectious stress factors such as brucellosis, which are prevalent (Khan *et al*., 2016). Postpartum anestrus is reported in 30-50% of crossbred cattle in India, which is a condition of silent heat, cystic ovarian degeneration (COD), or ovarian hypofunction caused by heat stress, poor forage quality and delayed involution of the uterus (Kumar *et al*., 2014). Cystic ovarian degeneration is characterized by the persistence of one or more large follicular structures (>25 mm) on the ovary for more than 10 days in the absence

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of a functional corpus luteum. Such ovarian abnormalities disrupt normal estrous cycles and reduce fertility. Timed artificial insemination protocols based on Gonadotropin-Releasing Hormone (GnRH) and Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) are commonly employed to restore ovarian cyclicity by synchronizing follicular development and ovulation.

Assessment of ovarian reserve and follicular activity has gained considerable importance in bovine reproductive management. Anti-Müllerian Hormone (AMH), a glycoprotein member of the Transforming Growth Factor-beta (TGF-β) family, is produced by granulosa cells of growing preantral and small antral follicles. Within the ovary, AMH regulates the recruitment of primordial follicles and modulates follicular responsiveness to Follicle-Stimulating Hormone (FSH), thereby helping to preserve the ovarian reserve (Visser *et al.,* 2006). As follicles grow and approach dominance, AMH production declines, allowing further follicular maturation and ovulation. Several studies have demonstrated a strong positive relationship between circulating AMH concentrations and antral follicle count (AFC) in cattle. Animals with higher AMH levels and AFC generally exhibit improved ovarian activity, greater oocyte recovery rates, enhanced embryo production and better reproductive performance (Mossa *et al.,* 2012; Sharma *et al.,* 2025a,b,c). Furthermore, because AMH concentrations remain relatively stable throughout the estrous cycle, a single blood sample can provide a reliable estimate of ovarian reserve and reproductive potential. This characteristic has made AMH a valuable tool for selecting donor animals for assisted reproductive technologies such as ovum pick-up (OPU) and *in vitro* embryo production (IVEP) (Mossa *et al.,* 2017; Mossa and Ireland, 2019; Koca *et al.,* 2024; Sharma *et al.,* 2025a,b).

Despite its recognized importance, information on AMH profiles in postpartum crossbred cows affected by true anestrus or cystic ovarian degeneration remains limited. Moreover, little is known about how changes in circulating AMH relate to real-time alterations in AFC during hormonal treatment. Therefore, the present study was undertaken to evaluate plasma AMH concentrations and ultrasonographically determined AFC in cyclic, anestrus, and COD-affected crossbred cows before and after a standard GPG synchronization protocol, and to assess the usefulness of AMH as a practical biomarker of ovarian response under field conditions.

Materials and Methods

Experimental Location and Climatic Conditions

The present study was conducted following approval by the Institutional Animal Ethics Committee of the College (No. VETCOLL/IAEC/2025/24/PROTOCOL-06) at the Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Sardarkrushinagar, Gujarat (India), in collaboration with Banas Dairy, Palanpur. The field component of the study was carried out from March to October 2025 in the milk shed area of Banas dairy. The study area is located between 24.258°-24.361° N latitude and 72.190°-72.359° E longitude at an altitude of approximately 145-152 meters above mean sea level. The region experiences a tropical semi-arid climate with an average annual rainfall of 1,346.1 mm. During the study period, the mean maximum and minimum temperatures were 34.3°C and 18.3°C, respectively, while relative humidity ranged from 51.1% to 72.7%.

Selection of Animals and Experimental Design

Thirty postpartum crossbred dairy cows (2^{nd} to 4^{th} parity) maintained under identical feeding, management, and environmental conditions were selected for the study. The reproductive status of each animal was assessed through per-rectal palpation and transrectal ultrasonography. Based on clinical and ultrasonographic findings, animals were allotted into three groups comprising 10 cows each:

Group I (COD): Cows diagnosed with cystic ovarian degeneration, characterized by the presence of a follicular cyst greater than 25 mm in diameter persisting for at least 10 days in the absence of a functional corpus luteum.

Group II (Anestrus): Postpartum cows showing no behavioural signs of estrus since calving and possessing small, inactive ovaries without functional follicles or luteal structures.

Group III (Cyclic): Apparently healthy cows exhibiting regular estrous cycles and normal ovarian activity, which served as the control group.

Synchronization Protocol and Breeding

All animals were subjected to a common GPG (Ovsynch) synchronization protocol. On Day 0, cows received 20 µg of GnRH (Buserelin acetate) intramuscularly. On Day 7, 500 µg of $PGF_2\alpha$ (Cloprostenol sodium) was administered intramuscularly, followed by a second GnRH injection (20 µg) on Day 9. Animals exhibiting induced estrus following treatment were inseminated using frozen-thawed semen obtained from a fertile bull. Pregnancy diagnosis was performed 60 days after artificial insemination by transrectal palpation and ultrasonography.

Ultrasonographic Examination and Antral Follicle Count

Ovarian examinations were performed on Day 0 and Day 9 using a portable B-mode ultrasound scanner equipped with a 7.0 MHz linear-array transducer. Instrument settings were maintained constant throughout the study to ensure uniform image quality. Before examination, the rectum was evacuated manually and the lubricated transducer was introduced transrectally. Both ovaries were systematically scanned from one pole to the other and all visible antral follicles measuring ≥3 mm in diameter were identified and recorded. The total antral follicle count (AFC) for each animal was calculated as the sum of follicles observed in both ovaries (Burns *et al.,* 2005).

Blood Collection and Estimation of Plasma AMH

Blood samples (10 mL) were collected from the jugular vein of each cow on Day 0 and Day 9 into EDTA-coated vacutainer tubes. Samples were transported to the laboratory under chilled conditions and centrifuged at 3,000 × g for 15 min at 4°C. The separated plasma was aliquoted into sterile microcentrifuge tubes and stored at -20°C until further analysis. Plasma AMH concentrations were determined using a commercially available bovine-specific ELISA kit (Puregene Bovine AMH Kit, 96T) following the manufacturer's instructions. A standard curve was generated using six AMH standards ranging from 25 to 800 ng/L and sample concentrations were calculated from the regression equation obtained from the standard curve.

Statistical Analysis

Data were analyzed using SPSS (version 22.0) and are presented as Mean ± SEM. Differences among experimental groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test. Changes within groups between Day 0 and Day 9 were assessed using paired t-tests. The relationship between plasma AMH concentration and AFC was examined using Pearson's correlation analysis. Estrus induction and conception rates were compared using the Chi-square test. Binary logistic regression and receiver operating characteristic (ROC) curve analyses were performed to assess the predictive ability of AMH for treatment response. Statistical significance was considered at $p<0.05$, while $p<0.01$ and $p<0.001$ were considered highly significant.

Results and Discussion

Ovarian Antral Follicle Count (AFC)

Baseline ultrasonographic evaluation revealed distinct differences in ovarian follicular activity among the experimental groups. Before treatment, anestrus cows exhibited a significantly lower total AFC (13.30±1.23) than cyclic (22.40±2.30) and cystic ovarian degeneration (COD) cows (23.10±2.64; $p<0.01$). The reduced AFC observed in anestrus animals reflects diminished follicular recruitment and ovarian inactivity, indicating a compromised ovarian reserve. Similar findings have been reported by Mahamadou *et al.* (2021), who described impaired follicular development as a characteristic feature of postpartum anestrus.

In contrast, cows affected with COD exhibited AFC values comparable to those of normal cyclic cows. This finding suggests that in spite of disrupted ovulatory mechanisms in COD, the recruitment and growth of small follicles remain largely intact (Rico *et al.,* 2009). Therefore, the persistence of ovarian cysts appears to be associated with failure of ovulation rather than an inability to recruit follicles. No significant difference was observed between follicle counts in the left and right ovaries ($p>0.05$), indicating that the reproductive disorders investigated in the present study were associated with systemic endocrine alterations rather than localized ovarian dysfunction.

Following GPG synchronization protocol, AFC increased significantly in all groups. The most pronounced response was observed in anestrus cows, where AFC increased from 13.30±1.23 to 28.70±1.71 ($p<0.001$). Post-treatment AFC values reached 32.40±2.65, 28.70±1.71and 31.50±2.94 in cyclic, anestrus and COD cows, respectively. This marked increase indicates successful recruitment of a new follicular wave and enhanced follicular growth following hormonal stimulation. Interestingly, the significant differences in AFC observed among groups before treatment were no longer evident after treatment ($p=0.468$). This finding demonstrates the effectiveness of the GPG protocol in restoring follicular activity and normalizing ovarian dynamics in both anestrus and COD affected cows. Similar observations have been reported by Morotti *et al.* (2017), who documented improved follicular recruitment following synchronization treatment.

Plasma Anti-Müllerian Hormone (AMH) Concentration

Changes in plasma AMH concentrations closely paralleled the ultrasonographic findings. Before treatment, anestrus cows exhibited lower AMH concentrations (229.50±24.18 ng/L) than cyclic (300.97±24.36 ng/L) and COD cows (301.97 ± 27.30 ng/L). Since AMH is secreted by granulosa cells of small growing follicles, the lower hormone concentration in anestrus cows directly reflects their reduced follicular population and diminished ovarian activity. Interestingly, AMH concentrations in COD cows were nearly identical to those observed in cyclic animals. This suggests that despite the presence of persistent follicular cysts, the recruitment of small antral follicles remains active. Similar observations have been reported in women with polycystic ovarian syndrome, where elevated follicular recruitment is associated with increased circulating AMH concentrations (Bungum *et al.,* 2013).

Following treatment, plasma AMH concentrations increased significantly ($p<0.05$) in all groups, reaching 416.50±24.56 ng/L in cyclic cows, 328.23±24.91 ng/L in anestrus cows and 364.83±30.84 ng/L in COD cows. The increase in circulating AMH concentrations suggests enhanced granulosa cell activity and expansion of the pool of small growing follicles following synchronization. Furthermore, the numerical differences in AMH concentrations among groups became considerably less pronounced after treatment ($p=0.084$), indicating that the GPG protocol successfully restored a comparable functional ovarian status across the different reproductive conditions. The concurrent increase in AFC and AMH confirms that hormonal synchronization stimulated folliculogenesis without adversely affecting follicular endocrine function. The level of AMH and ovarian reserve is affected by genetic and environmental variables,

including prenatal conditions and maternal nutrition, implying that hygienic population and genetic engineering can be used to improve fertility in the cattle population (Nawaz *et al.,* 2018; Mossa and Ireland, 2019).

Relationship between AFC and Plasma AMH

Pearson's correlation analysis demonstrated a strong positive relationship between AFC and plasma AMH concentration before (r=0.799; p<0.001) and after treatment (r=0.708; p<0.001) (Fig. 1). This finding confirms that circulating AMH accurately reflects the size of the active follicular pool and can serve as a reliable endocrine indicator of ovarian reserve. Similar correlations have been reported by Kekan *et al.* (2019), Guanga *et al.* (2022) and Sharma *et al.* 2025a,b,c), who documented strong associations between AFC and AMH in cattle and buffaloes.

Although the correlation coefficient declined slightly after treatment, the relationship remained strong, indicating that AMH continued to accurately reflect ovarian follicular activity even after hormonal intervention. The slight reduction in correlation may be attributed to temporary differences between rapid follicular growth and subsequent granulosa cell secretory responses during synchronized follicular wave development. The persistence of a strong positive association before and after treatment validates AMH as a practical and non-invasive biomarker of ovarian reserve. These findings support the use of AMH as an alternative to repeated ultrasonographic examinations, particularly under field conditions where access to advanced imaging facilities may be limited.

Estrus Response and Fertility Performance

Estrus response and conception rates differed numerically among groups following synchronization. Cyclic cows exhibited the highest estrus response (90.0%) and conception rate (66.7%), followed by COD cows (70.0% and 57.1%, respectively) and anestrus cows (60.0% and 50.0%, respectively).

The intermediate fertility response observed in COD cows indicates that cloprostenol-induced luteolysis and subsequent GnRH-mediated ovulation successfully resolved follicular persistence in the majority of affected animals. In contrast, the relatively lower response in anestrus cows may be attributed to delayed follicular maturation, silent ovulation and the gradual recovery of ovarian function typically observed during the postpartum period. Despite numerical differences, conception rates did not differ significantly among groups (p>0.05). This finding suggests that appropriate hormonal synchronization can effectively reduce the reproductive problems associated with anestrus and COD, enabling affected cows to achieve fertility outcomes comparable to those of normal cyclic animals.

Predictive Value of AMH for Treatment Outcome

To determine whether AMH could predict treatment success, logistic regression and ROC analyses were performed. Logistic regression indicated that increasing baseline AMH concentrations were associated with a positive trend toward conception; however, this relationship was not statistically significant (OR=1.004; p=0.397). Similarly, ROC analysis demonstrated only moderate predictive ability of AMH for treatment outcome (AUC=0.665). Although AMH accurately reflected ovarian reserve and follicular activity, its ability to predict final conception was limited. This observation is biologically expected because conception is a multifactorial process influenced not only by ovarian reserve but also by oocyte competence, uterine health, metabolic status, endocrine balance, semen quality and insemination timing. Therefore, while AMH serves as an excellent marker of the quantitative aspect of ovarian reserve, it should not be considered a standalone predictor of fertility.

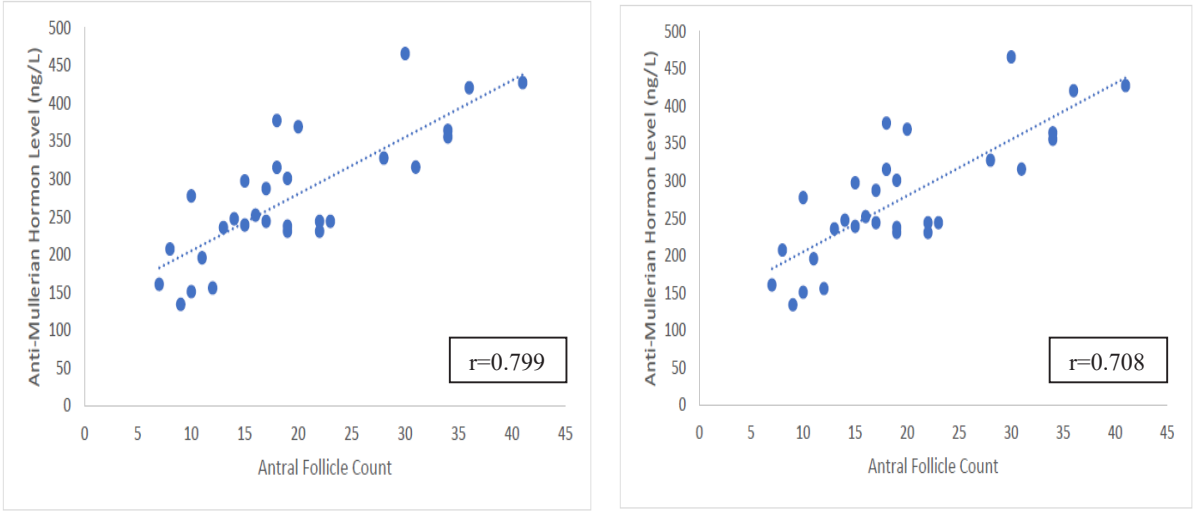


Fig. 1: Scatter plot showing relationship between total AFC and plasma AMH levels before treatment (r=0.799; p<0.001) and after treatment in crossbred cows (r=0.708; p<0.001)

Conclusion

The study demonstrated that postpartum anestrus cows possess a significantly lower antral follicle count (AFC) and numerically lower plasma Anti-Müllerian Hormone (AMH) concentrations compared to cyclic and COD-affected cows, indicating reduced ovarian reserve and follicular activity. The plasma AMH closely mirrors ultrasonographically determined AFC and reliably reflects changes in the active follicular population following synchronization therapy. A strong positive correlation between AFC and plasma AMH concentration was observed both before and after GPG treatment, confirming that circulating AMH reliably reflects the size of the active follicular pool in crossbred dairy cows. The GPG protocol effectively stimulated follicular recruitment and restored ovarian activity in both anestrus and COD-affected crossbred cows, highlighting its utility as a therapeutic approach for improving reproductive performance under field conditions. Although AMH showed a positive association with treatment response, its ability to predict conception was limited, indicating that fertility outcomes are influenced by multiple physiological and management-related factors beyond ovarian reserve alone. Overall, plasma AMH can serve as a practical and non-invasive biomarker for assessing ovarian reserve and monitoring ovarian response to synchronization therapy in crossbred dairy cows under field conditions.

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