Anti-Müllerian hormone, antral follicle count, and progesterone evaluation in Italian Mediterranean buffalo heifers

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SUMMARY

Anti-Müllerian hormone (AMH) has been used as a molecular marker of the ovarian follicular pool and follicular responsiveness to superovulation treatments in cattle and other species. Early studies in buffalo cows indicated that circulating AMH levels were relatively low, which appeared to be correlated with ovarian follicular reserve. This study aimed to evaluate AMH in buffalo heifers to investigate its potential correlation with the phase of the oestrous cycle and follicle count (FC). For this study, forty-two cycling Mediterranean buffalo (*Bubalus bubalis*) heifers, aged 18-20 months, were selected in a Sicilian farm. Using rectal palpation and ultrasound exam of the genital tract, recording uterine tone and ovarian findings (follicles and corpus luteum), the heifers were divided into two main groups: those in the luteal phase (n = 32) and those in the follicular phase (n = 10). Each ovary was carefully examined and the total number of follicles ≥ 3 mm in diameter was duly recorded for each animal. Blood samples were taken from the caudal vein for progesterone and AMH assay. The unpaired Wilcoxon signed-rank test was used to evaluate longitudinal changes in hormone levels from the follicular to the luteal phase. The Pearson correlation coefficient assessed the possible correlation between AMH and progesterone and between AMH and FC. The results indicated no significant difference in AMH levels between the follicular and luteal phases, and no correlation between AMH and P4. However, a significant correlation was observed between FC and AMH. AMH in buffalo heifers was not found to be correlated with the phase of the oestrous cycle, but rather with FC. The parallelism with bovine species suggests that AMH may be a useful indicator for selecting buffalo heifers with good fertility and long productive life, which could potentially serve as candidates for reproductive biotechnology.

KEYWORDS

Ovary; water buffalo; oestrous cycle, AMH; ultrasound.

INTRODUCTION

The Italian Mediterranean buffalo has been officially classified as a *Bubalus bubalis* var. *bubalis* breed since 2000. It has been documented that the breed originated in Italy during the Roman era [1]. Water buffalo is a short-day breeder species. The oestrus cycle lasts between 20 and 22 days, with 18 to 26 variations [2]. The oestrus lasts between 12 and 30 hours, with ovulation occurring 11 to 18 hours after the end of the oestrus period [2]. The oestrus signs are almost absent in buffalo cattle [3]. The gestation period is 310-330 days. The utilisation of reproductive biotechnologies in water buffalo presents certain challenges. Embryo therapy may offer promising applications in these species [4]. However, the *in vivo* production of embryos is difficult. It has been demonstrated that, in contrast to the bovine species, water buffalo cows exhibit a lack of responsiveness to superovulation protocols [5;6]. It is likely that water buffalo possess a reduced follicular pool in comparison with cattle, which would result in a lower overall embryo number (typically 1-2) [7]. This phenomenon may be attributed to the observation of a greater number of atretic follicles during follicular waves in water buffalo compared to cattle [8;9]. The number of ovarian follicles and oocytes, or the follicular pool, is determined during the gestation period. This decline is associated with age and is not replenished following parturition [10]. Most existing studies have demonstrated a positive correlation between the size of the ovarian reserve and the fertility potential of female cattle. Furthermore, the validation of two size markers of the ovarian reserve in cattle has been conducted: the recruited follicles number during follicular development waves and peripheral concentrations of the Anti-Müllerian hormone (AMH) [11;12]. AMH is a glycoprotein produced exclusively by granulosa cells of developing follicles in females [13, 14]. Some studies demonstrated that AMH concentrations exhibit minimal variation during oestrous cycles in cattle [11]. In dairy cows, AMH concentrations remained static during the same oestrous cycle [15; 16], on different days of two oestrous cycles [15], and within the same individual during natural and synchronised oestrous cycles [17]. These findings suggest that AMH concentrations can be accurately determined with a single blood sample taken at random on any day of the cycle in adult cattle. The overall mean AMH concentration during ovulatory follicular waves per animal was found to be significantly correlated with the mean peak FSH concentration during the two or three waves of the oestrous cycle [18]. Additionally, a positive correlation was identified between the follicles number and AMH in Holstein, Gyr, and Murrah cattle [19], substantiating the reliability of both Follicle Count (FC) and AMH as biomarkers for predicting the size of the ovarian reserve in age-matched cattle. To date, there are few studied on AMH evaluation in water buffalo, especially for Mediterranean Italian buffalo. Liang et al. have focused on the detection of AMH in the follicular fluid of ovaries obtained from slaughtered animals [20]. In this study, it was demonstrated that the AMH concentration decreased in conjunction with an increase in follicular diameter [20].

The objective of this investigation was to investigate AMH production in Mediterranean water buffalo heifers during the oestrus cycle phase and the eventual correlation with antral follicle number.

MATERIALS AND METHODS

This study was preliminary approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Messina. A total of 42 water buffalo heifers of the Mediterranean Italian Buffalo breed were evaluated. The heifers were 18 to 20 months of age and represented the restocking of a herd in Ragusa, Italy. A reproductive examination was conducted via rectal palpation to evaluate ovarian function (corpus luteum/follicles), uterine tone, and discerning diseases of the reproductive tract or pregnancy. The clinical findings were subsequently supported through ultrasound examination (Fujifilm SonoSite M Turbo; linear endo cavitary probe 7.5 MHz). Cases of endometritis, ovarian cysts and anoestrous were excluded. Subsequently, the heifers were classified into two groups based on the findings of the oestrus cycle: the follicular phase and the luteal phase. An ultrasound examination was employed to conduct a comprehensive analysis of each ovary, with the aim of counting antral follicles [18]. Each ovary was scanned in its entirety to ascertain the position of follicles and corpus luteum. The various images of the ovarian section were recorded and the position of the antral follicle ($\geqslant$ 3 mm) and corpus luteum were marked on an ovarian map (Figure 1), along with the total number of antral follicles. A blood sample was collected using a 10ml syringe and a 21G needle from the caudal vein. The blood samples were stored in test tubes containing sodium citrate-like coagulation activator and refrigerated in a box at 4°C before being transported to the laboratory. In-laboratory, the blood samples were subjected to centrifugation at 3500 rpm, after which the serum was transferred to 3 Eppendorf cuvettes. The evaluation of progesterone (P4)

was conducted using the Speed Progesterone immunochromatographic test (Virbac, Carros, France). AMH was evaluated using an enzyme-linked immunosorbent assay (ELISA) system with a specific bovine kit (Bovine AMH ELISA-AL-114®, Ansh Labs, Webster, TX, USA). The sensitivity of the test was 11 pg/ml, and the intra-assay coefficient of variation was less than 5%.

The resulting data were subjected to statistical analysis. The longitudinal changes in hormone levels, from the follicular phase to the luteal phase, were evaluated using the Wilcoxon test for unpaired data. The correlation between AMH, P4 and FC was evaluated using the Pearson correlation coefficient. A *p*-value of less than 0.05 was statistically significant.

RESULTS

The results of the clinical screening indicated that 10 heifers were in the follicular phase (proestrus/oestrus) and 32 were in the luteal phase (metaestrus/diestrus). FC was observed to range between 3 and 22, with a median value of 13. The P4 values were observed to range between 0.2 ng/ml and 6 ng/ml, with a median value of 3.6 ng/ml. The AMH values were observed to range between 2.08 pg/ml and 160.97 pg/ml, with a mean of 12.5 pg/ml. The data were subsequently organised into two distinct groups for subsequent analysis. The median values for the follicular phase in water buffalo (n = 10) were: FC=13, AMH=28.41 pg/ml, and P4=0.65 ng/ml. The median values for the luteal phase (n = 32) were: FC=13, AMH=13.35 pg/ml and P4=3.6 ng/ml.

The Wilcoxon test demonstrated a statistically significant difference in progesterone levels ($p = 0.000001$), but no significant difference in FC and AMH levels ($p = 0.31$; $p = 0.15$). The discrepancies can be readily discerned through graphical representation, as illustrated in the boxes and plot graphs (figure 2). Pearson's correlation test did not reveal a statistically significant relationship between FC or AMH and P4 ($r = 0.28$, $p = 0.25$). Nevertheless, a significant correlation was identified between FC and AMH (Pearson's $r = 0.782$, $p = 0.000076$) (figure 3).

DISCUSSION

The present study aimed to evaluate the association between AMH, FC and P4 levels in the Mediterranean Italian buffalo. The dearth of studies on this species provided a foundation for analysis that was both intriguing and informative. The water buffalo displays a temperament like that observed in beef cattle. Despite their apparent docility, wild behaviour is primarily exhibited during handling procedures, particularly in younger animals. The water buffalo is a seasonal breed, with a breeding cycle that is optimised for reproduction during the autumn season (short-day breeder) [2]. Nevertheless, the animals' cyclicity could be extended throughout the year with effective herd management.

In our study, a single ovarian ultrasound examination was conducted following standard practice for beef heifers [21], to count antral follicles. The data obtained from each scan were recorded and subsequently mapped. It seems probable that the follicular count could be increased by identifying the onset of the follicular wave, before its deviation and the onset of the dominant phase. Synchronising oestrus or conducting a daily ovarian ultrasound examination over ten days may facilitate this process. However, the behaviour of the animal, the compliance of the farmer and the ethical risks involved have not been sufficiently analysed. The data obtained by FC (median=13) are comparable to those reported in the literature. Indeed, Liang et al. reported that the FC in the ovaries of slaughtered Mediterranean water buffalos was 12 [20], whereas the FC was 26 in Murrah buffalos [19]. In our study, some animals had a FC of 26, while others had only 3 antral follicles measuring 3 mm. It was established that FC does not fluctuate between the follicular and luteal phases of the oestrus cycle. These limited data suggest that *Bubalus bubalis* has a lower follicle number than *Bos taurus*, and even less so than *Bos indicus*, which is characterised by a large ovarian reserve [19]. It would therefore appear prudent to select animals with a superior follicular pool in this species. Furthermore, it was demonstrated that animals with an enhanced FC exhibit greater productivity than those with a markedly reduced FC [21; 22].

Progesterone was quantified using a commercially available canine-specific kit (Speed Progesterone, Virbac, Carros, France). Nevertheless, the progesterone molecule is identical across species, and the kit's calibration range (0-20 ng/ml) includes the typical values observed in ruminants. Therefore, the reliability of our methodology in the context of Mediterranean water buffalo was corroborated by the correlation between clinical signs, P4 values and existing literature. In this species, the concentration of haematic progesterone is basal during the follicular

phase and significantly elevated during the luteal phase (5-6 ng/ml) [23]. As anticipated, no correlation was identified between FC and progesterone levels. Instead, there seems to be a linear relationship (p=0.06) with a parallel line to AFC's median value, to demonstrate now that FC is the same during oestrus cycle. The ELISA kit employed for the analysis of anti-Müllerian hormone (AMH) was a bovine-specific AMH kit (Bovine AMH ELISA-AL-114®, Ansh Labs, Webster, TX, USA). In their study, Liang et al. employed a human kit [20]. The AMH values observed in water buffalo species are notably low in comparison to bovines [20]. In our study a median of 18 pg/ml and a range from 2 to 160 pg/ml was found. This value is lower than the value of 180 pg/ml reported in Murrah water buffalo [19], but Redhead et al. obtained AMH values lower than 80 pg/ml in most animals [24]. Accordingly, the data presented here agree with the findings of previous research in the field and can be assumed to reflect a genuine distinction between breeds. A further challenge arises from the presence of very low values approaching the lower limit of the kit's reading range (11 pg/ml). It can be stated with certainty that the Mediterranean water buffalo exhibits a markedly low AMH haematic concentration, which is indicative of a small ovarian reserve. In this farm, only a few animals have haematic AMH concentrations over 100 pg/ml. These animals also display a greater ovarian reserve, confirming the strong relationship between AMH and FC observed in previous studies.

Blood AMH concentration is not correlated with P4, so there is no correlation between AMH in follicular phase vs AMH in luteal phase. This data is in according to literature [11;15;17]. Even in human species some studies showed that AMH concentration don't change during the same menstrual cycle [25; 26; 27; 28]. This data supports the choice to use a single blood sample in an any day of oestrus cycle to assay AMH in buffalo species.

CONCLUSION

In according to the literature, the present study suggests that the determination of the serum AMH in water buffaloes can be made with a single blood sample taken at any time during the oestrus cycle. Serum AMH has been proposed as a marker of ovarian reserve, which is associated with several productive and reproductive traits. Therefore, in the water buffalo, which is classified as a species with low ovarian reserve, AMH can be used to identify the most productive animals.

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AUTHOR CONTRIBUTIONS INVESTIGATION

writing original draft SM; Conceptualization GM; Investigation GM, MLS, FA; Writing/review and editing GC; GM; SM; Formal analysis GC; GM; SM. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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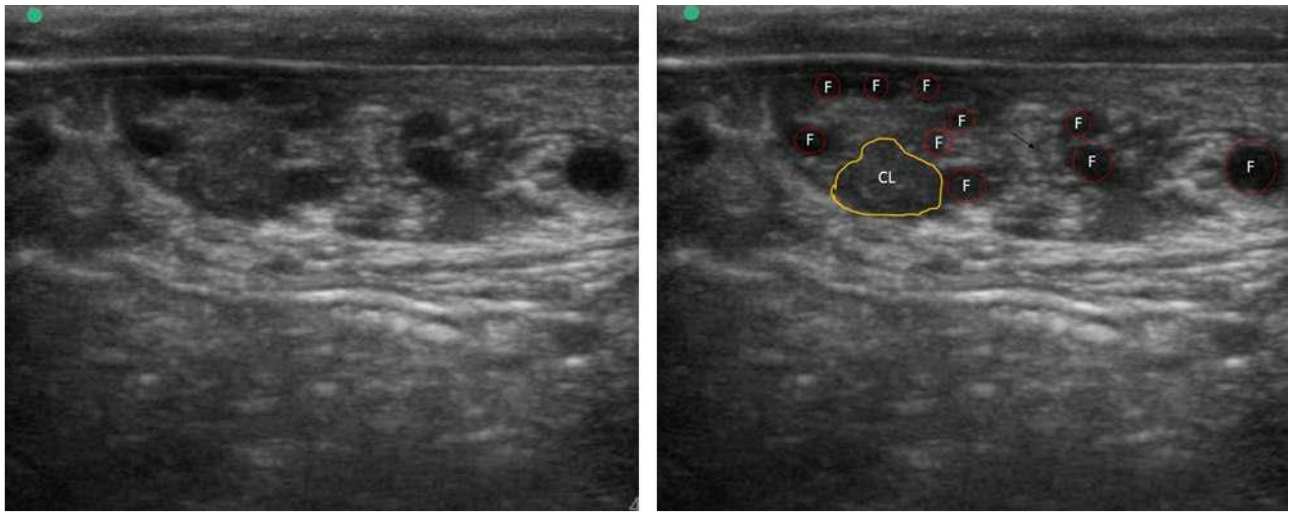

Figure 1 Ultrasound image and ovarian map for Follicle Count (FC)

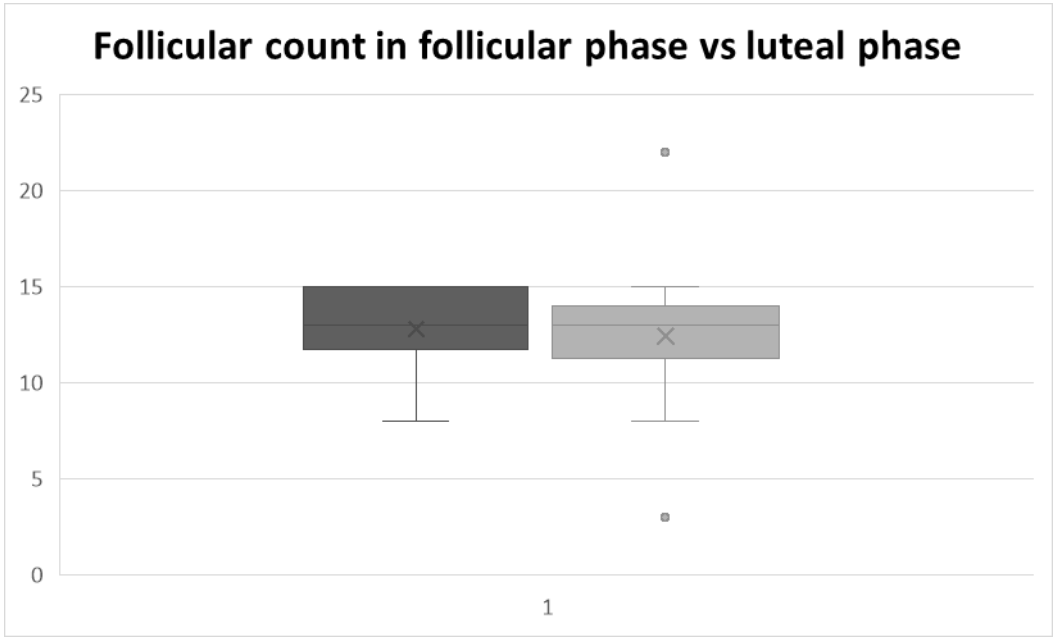

Figure 2 Boxes and wishers plot showing FC difference between follicular phase and luteal phase

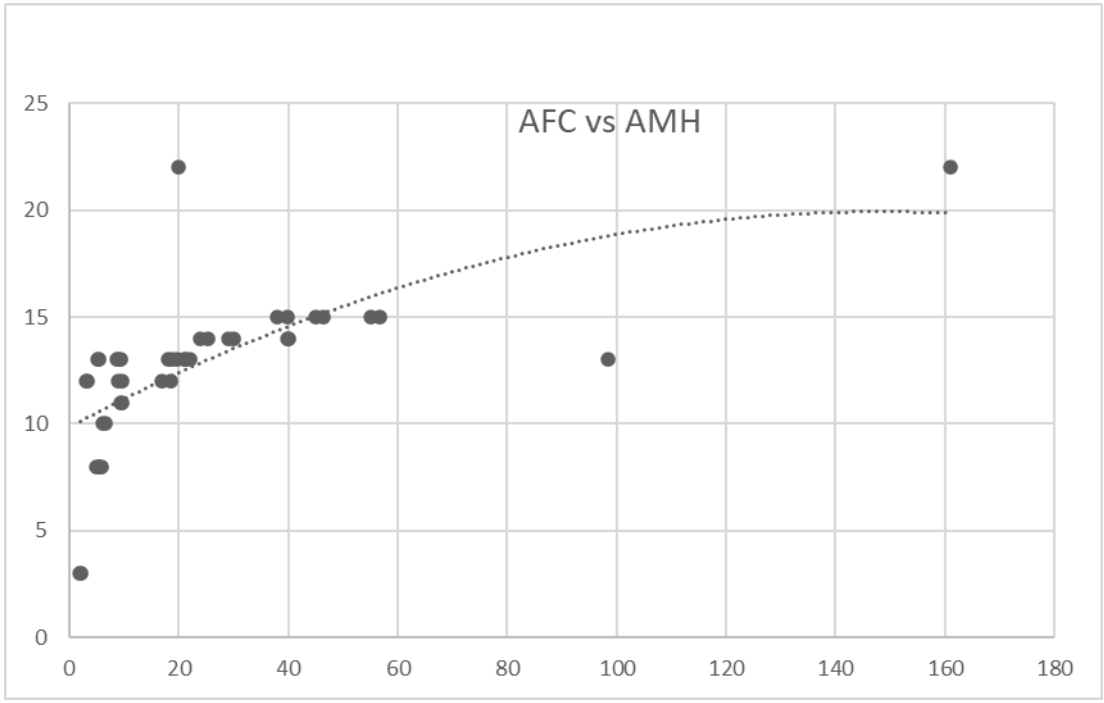

Figure 3 Strong correlation (p=0.000076) between anti-Müllerian hormone (AMH) and follicle (>3 mm) count (FC). X axis: AMH (pg/ml). Y axis: follicular count.