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RESEARCH ARTICLE

Changes in Serum Levels of Anti-Mullerian Hormone and Ovarian Steroids in Barki Sheep during Follicular and Early Luteal Phase of Estrous Cycle

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ABSTRACT

Although Anti-mullerian hormone (AMH) has been extensively studied in ruminants in the context of assisted reproductive techniques (ART), few data are available on its physiological levels during estrous cycle in adult ewes. Therefore, the aim of the current study was to investigate the daily secretion pattern of AMH, progesterone (P4), estradiol17-β (E2) during follicular and early luteal phase in cyclic Barki ewes. After confirmation of cyclicity using ultrasonography, a group of ewes (n=10) were treated with GnRH-7ds-PGF2α to synchronize their ovarian activity. Blood samples were collected and ultrasonographic scanning of ovaries was performed 2 days before PGF2α injection, on the same day of its injection (D 0), then continued daily until day 7 after treatment. Changes in serum levels of AMH, P4, and E2 were determined and number of pre-ovulatory follicles (2-5mm diameter) was recorded. Results showed that AMH levels decreased after PGF2α injection with lowest significant concentrations (0.86±0.05 ng/ml) were recorded on day 2 ($P<0.01$). Whereas it increased on day 3 until day 5 then declined again thereafter. Serum AMH levels were positively correlated ($P<0.001$) with number of small/medium antral follicles and P4 ($r=0.88$, and $r=0.41$ respectively), while it was negatively correlated with E2 ($r=-0.74$, $P<0.001$). In conclusion, the presented work showed that AMH followed a dynamic profile during follicular and early luteal phase of adult ewes. The noticed fluctuations in AHM level could hold a clinical usefulness in reproductive management programs of sheep, as low AMH levels were associated with terminal follicular growth. Accordingly, an optimized estrous synchronization protocols can be designed.

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INTRODUCTION

The role of endocrine signals in regulating follicular development has been long established. On the other hand, several studies over the past years have shown that intra-ovarian factors also play a critical role in determining follicular fate. Accumulating evidence validated that anti-mullerian hormone (AMH) is one of those factors controlling folliculogenesis (Monniaux *et al*., 2014). AMH is a glycoprotein that belongs to transforming growth factor β (TGFβ) family, it was first recognized for its role in embryonic development as inhibitory factor of Mullerian duct in male fetus (Jost *et al*., 1973). AMH is not expressed in ovaries of female fetuses; however few days after birth, it can be detected only in granulosa cells of primary and small growing follicles (Durlinger *et al*., 2002). It was demonstrated that AMH knockout mice show a premature depletion of primordial follicle pool (Durlinger *et al*., 1999). Moreover, in vitro studies indicated that AMH has reduced estrogenic capacity of FSH-stimulated granulosa cell culture in sheep (Campbell *et al*., 2012). Likewise, a decreased aromatase activity and LH receptor expression was detected in rat and pig granulosa cell cultures when treated with AMH (DiClemente *et al*., 1994) . Those results indicate a wide role of AMH in both early and terminal stages of follicle growth. Abnormal high AMH secretion has been associated with polycystic ovary syndrome (PCOS) in women (Fallat *et al*., 1997), and granulosa-theca cell tumors (GTCTs) in cattle (El-Sheikh Ali *et al*., 2013). Currently, AMH is used as fertility predictor and endocrine indicator of the ovarian pool of growing follicles in women undergoing IVF programs (Visser and Themmen, 2005). Further studies have shown

that AMH is a reliable indicator of capacity potential of embryo donors in multiple ovulation and embryo transfer (MOET) programs in ruminants such as cows (Rico *et al*., 2012), goats (Monniaux *et al*., 2011), and sheep (Torres-Rovira *et al*., 2014; Qureshi *et al*., 2015).

Several hormonal protocols were used to induce and synchronize estrous in ewes combined with timed artificial insemination to increase lambing rate and to shorten breeding periods. However, the general fertility outcomes of these protocols are not satisfying, which is presumably due to the lack of control of follicle development (Husein and Kridli, 2003). Noticeably, not much advances were introduced to optimize synchronization programs used in sheep over the past 20 years. Improved knowledge of ovarian dynamics considering the role of intra-ovarian signals will allow us to update and enhance reproductive management programs in this species. At present, there is lacking data about physiological levels of AMH during estrous cycle of adult ewes. Therefore, the aim of the current study was to monitor daily secretion pattern of AMH, P4, and E2 and to characterize the relationship between those ovarian hormones and the number of small/medium size follicles of 2-5 mm in diameter during follicular and early luteal phase in cyclic adult ewes.

MATERIALS AND METHODS

Animals: The current study was conducted in the facility of Faculty of Veterinary Medicine, Alexandria University. Ten sexually mature Barki ewes of ages between 2 to 3 years weighing about 45-50 kg with history of normal breeding record were used for the experiment. Animals were maintained at ambient temperature and natural day length in covered pens with adjacent yard. They were fed totally mixed ration and green roughages with ad libitum access to water and mineral blocks. The experimental procedures were approved by local ethical committee of Alexandria University; all the procedures were performed in accordance with the guidelines for the care and use of agricultural animals for research.

Synchronization of ovarian activity: The experiment was conducted during the breeding season (Mid-October, 2016); cyclicity of animals was confirmed by ultrasonography. Ovarian activity of the selected ewes was synchronized according to Martinez and co-workers (Martinez *et al*., 2015) with modification (P4 administration was omitted and a lower dose of Buserelin was used). Treatment included i.m injection of 20µg Buserelin-GnRH analogue; Receptal® (Intervet Corp. /Schering-Plough animal health, USA), followed 7 days later by i.m injection of 263µg of Cloprostenol-PGF2α analogue; Estrumate® (Intervet Corp. /Schering-Plough Animal Health, USA).

Blood sampling: To monitor ovarian activity in synchronized ewes, blood samples were collected 2 days (-2) before PGF2α injection, just before injection (D 0), with another samples collected 8 hours later. Thereafter, daily blood samples were collected until day 7 after treatment. sampling was scheduled at the same time early morning, blood was withdrawn from the jugular vein into plain tubes; serum was separated after centrifugation for 15 min at 1000x g and stored at -20˚C for later assay of AMH, P4, and E2.

Ultrasonography of ovaries: Trans-rectal ultra-sonographic scanning of ovaries was performed on -2, day 0, then daily until the end of the experiment. Scanning was performed using real time B-mode scanner (AMIB7V, Noveko echographs. Inc. Canada) connected to dual frequency (2.6-6.0 MHz) linear array transducer. Ovaries were located by careful scanning in lateral directions to the urinary bladder and uterine horns. Number of small and medium sized antral follicles (2-5 mm in diameter) was determined. Antrum size of follicles was estimated by measurement of its diameter when the antral cavity appeared to be at its maximal size during scanning.

Hormonal assay: Serum concentrations of AMH were measured using sheep (MIS/AMH) quantitative sandwich ELISA kit (MBS01008-MyBiosource, Inc., USA). Sensitivity of the kit was 0.1ng/ml with intra-assay CV<15%. Serum concentrations of P4 (P4) were determined by RIA (RIA P4, IMMUNOTECH, Czech Republic) validated previously for ovine use (Titi *et al*., 2008). E2 concentrations were detected using RIA kit (Orion Diagnostic Corp, Espoo, Finland) validated for ovine use (Torres-Rovira *et al*., 2014), Sensitivity of the kit was 0.5 pg/ml and the intra-assay CV<10% for both of the steroids. All samples were run in one assay; all analysis procedures were performed following instructions provided by the manufacturers.

Statistical analysis: The effect of time on hormone concentrations were analyzed by repeated measures analysis of variance (ANOVA) using the General Linear Model procedure of SAS 2002 (SAS, 2002). All variables were assessed for normality (PROC UNIVARIATE NORMAL PLOT of SAS), and transformed to the log10 scale, if needed, to normalize the distribution before running ANOVA. Day of PGF2α injection (D 0) was used as baseline for comparing contrast variables over time. For correlation, data was analyzed using Pearson correlation coefficient, and simple linear regression analyses were performed using the hormone concentration as a predictor variable, and the number of follicles as a response variable. The data are expressed as means ± standard error (SE).

In order to characterize hormonal patterns, data was assigned to certain time periods of physiological importance within which meaningful results could be used to quantify differences. The time periods were: 1) late luteal phase of the preceding ovulatory wave, from (-2 day) until (D 0); 2) follicular phase, 8 hours after PGF2α injection to the time E2 reached a maximum level (D 2); 3) early luteal phase, from day 3 (expected time of luteinization) until end of blood sampling (D 7).

RESULTS

The basis for using GnRH- PGF2α protocol to synchronize ovarian cycle is that exogenous GnRH given to cyclic ewes at random stages of their estrous cycle induces a LH surge that evokes ovulation or atresia of the

dominant follicle thus inducing follicular turnover (Webb *et al*., 1992). An interval of seven days between GnRH and PGF2α injection instead of standard 5 days protocol (Titi *et al*., 2008) will allow emergence of two follicular waves and development of luteal tissue by the time of PGF2α injection and hence a high precision of synchronization (Martinez *et al*., 2015).

Data of 3 ewes were removed from the analysis because they did not develop corpus luteum 2 days before PGF2α injections (P4<1ng/ml). In the remaining animals, serum concentrations of P4 on the day of PGF2α injection (D 0) were 15.01±0.48 ng/ml, which declined significantly (P<0.01) after induction of luteolysis to basal levels on day 1 where they remained low until day 3. An observed increase (P<0.05) was noticed on day 4 indicating a functioning corpus luteum (Fig. 1-C), the increase continued until the end of blood sampling (Fig. 1-A). During late luteal phase serum concentrations of E2 were 1.57±0.20 pg/ml, which started to increase (P<0.01) progressively 8 hours after induction of luteolysis that reached maximum levels (20.81±0.35 pg/ml) on day 2 indicating terminal follicular growth. Afterwards, E2 concentrations declined steadily to lowest level (P<0.01) at days 5 and 6 coincident with early luteal phase (Fig. 1-B). From late luteal to follicular phase AMH showed progressive decline (P<0.01) until day 2 when it recorded lowest concentration of 0.86±0.05 ng/ml. With the beginning of early luteal phase, serum AMH levels increased to 8.55±0.36 ng/ml on day 4 (P<0.05), while maximum concentrations were observed on day 5 (11.8±0.28 ng/ml) then it declined again thereafter (Fig. 1-A). A similar trend of change in the number of small/medium antral follicles was observed, mean follicles number was 9.7±0.56 before induction of luteolysis, which significantly declined (P<0.01) to lowest value recorded at 4.0±0.30 on day 2. Whereas during early luteal phase, follicles number increased gradually (Fig. 1-D) to record the highest value on day 5 (12.0±0.43), then declined thereafter. Serum AMH levels were positively correlated (P<0.001) with number of small/medium antral follicles and P4 (r=0.88, and r=0.41 respectively), while it was negatively correlated with E2 (r=-0.74, P<0.001). Relationship between AMH, E2 and number of follicles is shown in Fig. 2.

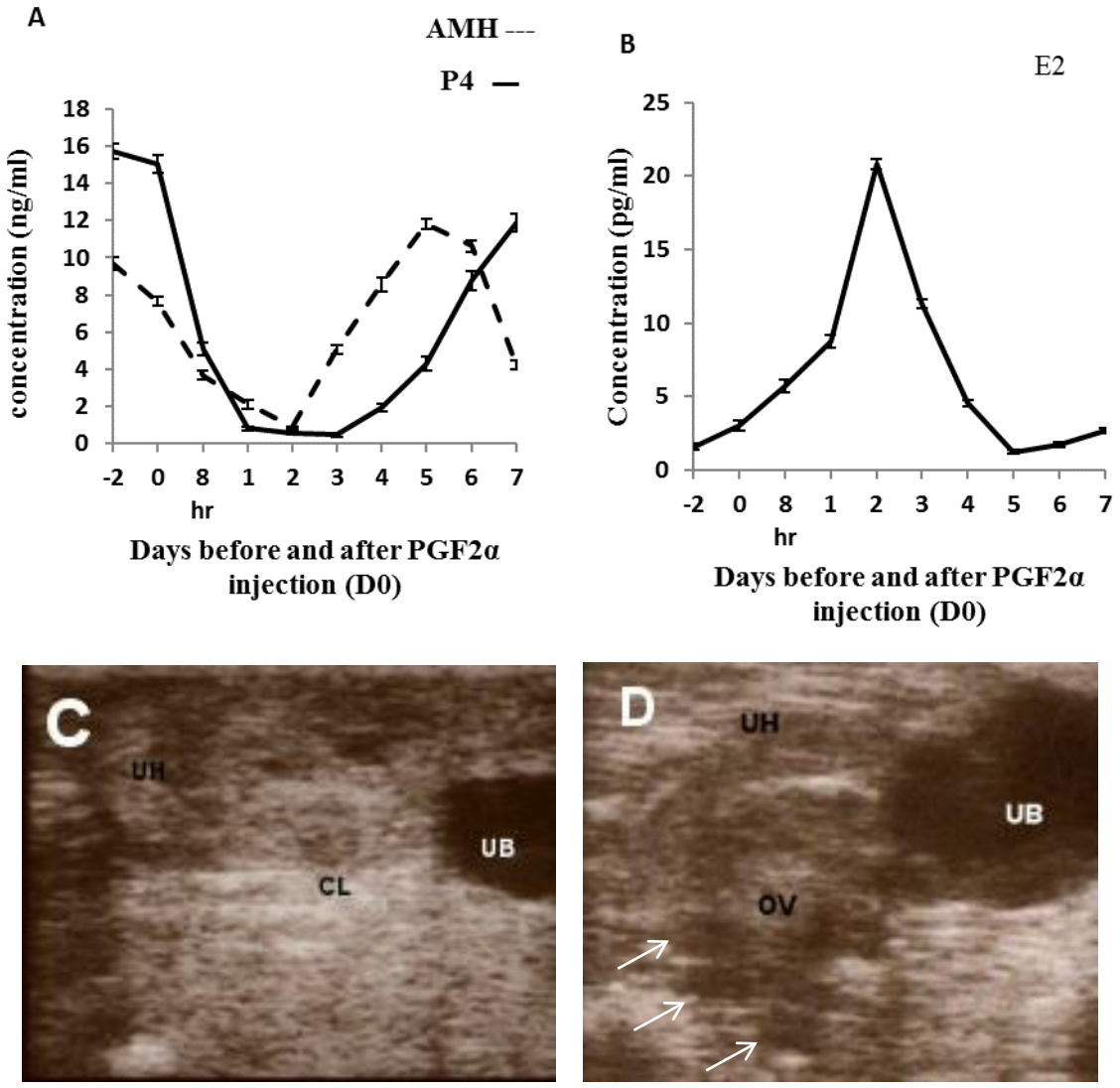

Fig. 1: Mean values (± SEM) of serum concentrations of P4, AMH (A) and E2 (B) during follicular and early luteal phase of estrous cycle in of adult ewes (n=7). Day of PGF2α injection is day 0. Sonographic scanning of the ovary on day 4 revealed the presence of corpus luteum "CL" (C). On day 5, multiple small/medium size follicles were observed "arrows" (D), UB: urinary bladder, UH: uterine horn, OV: ovary, images recorded using 4 MHz trans-rectal probe.

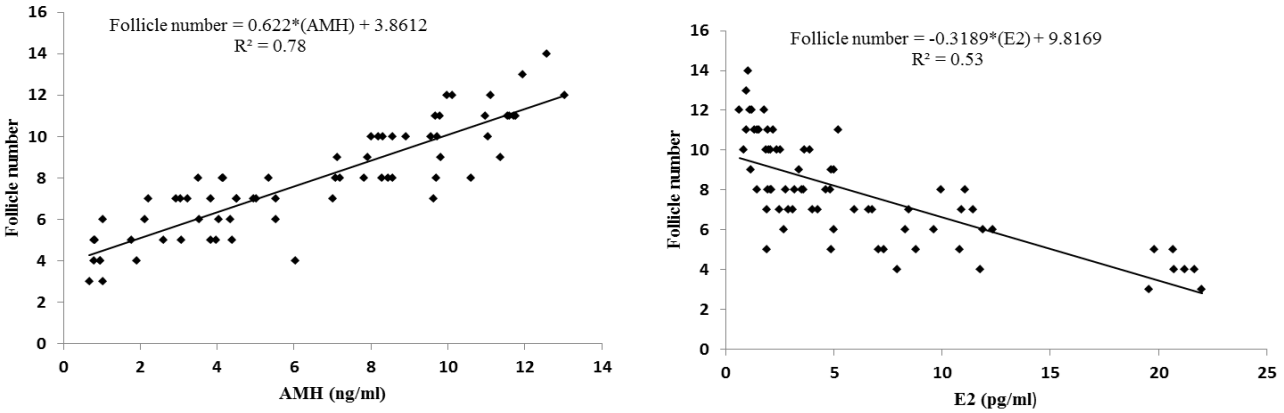

Fig. 2: Relationship between AMH, E2 and number of pre-ovulatory follicles (2-5mm in diameter). Follicles were counted using ovarian ultrasonography, each square represent data from one animal (n=7).

DISCUSSION

The focus of the current work was to determine daily pattern secretion of AMH, E2, and P4 by the ovary and to relate them with the change in the number of pre-ovulatory follicles (2-5 mm in diameter) during follicular and early luteal phase of estrous cycle in adult Barki ewes. Barki sheep are broadly raised in subtropical regions for their capability to resist harsh conditions. This breed were reported to have the ability to breed all year round with average estrous cycle length of 19.85 days (Sabra and Hassan, 2008). Results of the present study have shown that the size of the pool of pre-ovulatory antral follicles is significantly correlated with AMH serum levels. This finding is in line with data described previously on immunolocalization of AMH in ewe's ovary, which is strongly expressed in granulosa cells of all growing stages of follicle development (preantral and growing antral) with a marked decline with increasing follicle size (Campbell *et al*., 2012). Similar result was reported in goats, as single measure of AMH level during embryo collection session was highly correlated with the number of 1-5mm follicles (Monniaux *et al*., 2011). In prepubertal ewes, it was elucidated that small antral follicles of 2mm in size contribute the most to plasma AMH levels (Torres-Rovira *et al*., 2014), whether or not this rule applies to adult ewes yet remains to be established. In sheep, follicles between 2 and 5 mm in diameter are known to be gonadotropin-dependent (Monniaux *et al*., 2014). On the other hand, below the diameter of 2mm follicles constitute a pool of gonadotropin-responsive follicles. In this species, AMH was found to regulate the rate of follicles progression to gonadotropin-dependent phase (Campbell *et al*., 2012)

During the time-course of the experiment AMH exhibited a dynamic profile, initial concentrations were 9.69±0.34 ng/ml 2 days before PGF2α injection (late luteal phase of preceding cycle), then it declined significantly ($P<0.01$) 8 hours later and over the following 2 days (follicular stage after induced luteolysis). Notably low E2 concentrations were recorded before induction of luteolysis despite the presence of considerable number of small/medium sized follicles (9.71±0.56). Likewise, this remark was also reported by Souza and co-workers; the authors explained that estrogenic capacity of pre-ovulatory follicles is under control of luteinizing hormone (LH) pulses which are infrequent during late luteal phase (Souza *et al*., 1997). The concomitant decrease in AMH and increase in E2 levels at the transition between late luteal and early follicular stage observed in the current study is compatible with wave-like pattern of antral follicle development reported in sheep (Evans *et al*., 2000) and also support the inverse relationship of AMH with aromatase "CYP19A1" (Campbell *et al*., 2012), the enzyme that converts androgens to estrogens in granulosa cells. These results also could indicate and characterize selection and deviation of dominant follicle, when it acquires increasing capacity for E2 secretion. This concept is supported by the fact that AMH mRNA levels was found to decrease in both dominant and subordinate follicles around the time of follicular selection and deviation in cattle (Ilha *et al*., 2016) and human (Jeppesen *et al*., 2013).

The mean number of small/medium sized follicles was observed to be declining ($P<0.01$) as E2 concentrations were increasing during follicular phase. It was reported previously that number of medium sized follicles (4 mm) decline during approaching ovulatory wave in cyclic ewes (Toosi *et al*., 2010). These follicles apparently could not sustain its viability due to insufficient levels of circulating FSH induced by increasing concentrations of inhibin and E2 produced by the dominant follicle, eventually they undergo atresia (Tesone *et al*., 2009). In addition, dominant follicles were found to produce atretogenic factors that cause atresia of neighboring or subordinate follicles (Vitale *et al*., 2002). Atretic follicles have low intensity of AMH immuno-staining and express less AMH mRNA than healthy ones (Rico *et al*., 2011; Ilha *et al*., 2016) this possibly correlates to the further decrease in circulating AMH during terminal follicular development in ewes under present investigation. Typically, a peak in serum E2 concentrations occurs around the end of the growth phase of the dominant follicle in the wave (Driancourt, 2001), which in the current experiment was observed at day 2 after induced luteolysis. This matches findings of Martinez and co-workers as they reported largest size of ovulatory follicle 2 days after PGF2α injection (Martinez *et al*., 2015). At this point AMH recorded its lowest level (0.86±0.05ng/ml); concurrently P4 was at its basal levels that remained low until day 3. Thereafter, a steady increase of both AMH and P4 along with the number of

small/medium follicles was evident. This physiological event implicates ovulation/ follicular luteinization that mark early luteal phase. These observations are in agreement with the reported simultaneous increase of AMH and P4 during luteal phase of women subjected to controlled ovarian hyperstimulation (Fanchin *et al*., 2005). The observed increasing numbers of small/medium follicles at this point suggest that a new follicular wave has emerged, even though follicle stimulating hormone (FSH) secretion is known to be low during early luteal phase (Driancourt, 2001; Nett *et al*., 2002). In agreement with this finding, former studies on wave dynamics in ewes indicated that new follicular waves emerge during early luteal phase and they occur independently of FSH fluctuations (Ginther *et al*., 1995; Toosi *et al*., 2010); also they are accompanied by an increase in P4 secretion (Souza *et al*., 1998). According to the data available in cow and sheep, FSH in low concentrations and bone morphogenetic proteins (BMPs) produced by the oocyte and theca cells were shown to enhance AMH synthesis in granulosa cells of preantral and small antral follicles (Monniaux *et al*., 2012). Those stimulatory signals can partially explain the observed increasing levels of AMH during early luteal phase in the current study, because other paracrine and autocrine factors such as activins, inhibin and Insulin-like growth factor-I (IGF-I) also regulate follicular growth (Tesone *et al*., 2009) and consequently AMH secretion. IGF-I was found to modulate in-vitro action of AMH on FSH-stimulated granulosa cells in ewes (Campbell *et al*., 2012). Unlike ewe, AMH in cow declined rapidly after estrus and remained low between days 4 and 8 of the cycle then it increased slowly until next estrus (Rico *et al*., 2011). This could be accounted for by the shorter duration of estrous cycle in ewe with an average of 17 days for most of sheep breeds, also follicular growth pattern in sheep appears to be more dynamic than in cattle (Souza *et al*., 1997). Ovarian cycle in ewe exhibits 3 to 4 follicular waves with an average interweave interval of 3 to 5 days (Ginther *et al*., 1995). In the examined ewes herein, serum AMH declined again after day 5, which could be suggestive of degeneration /atresia of recruited follicles and the end of the first follicular wave (anovulatory wave) in the luteal phase.

Conclusions: Results of the current study expand our knowledge about endocrine background of follicular development in ovary of adult ewes. Serum AMH was found to follow a dynamic profile during follicular and early luteal phase of the ovarian cycle. Low AMH levels were recorded during terminal follicular growth, while was increasing at the time of wave emergence, its positive association with the number of growing pre-ovulatory follicles supports its ability as a reliable marker of antral follicles' pool size. The noticed fluctuations in AHM level could hold a clinical usefulness in determining efficacy of different estrous synchronization protocols. AMH measurement 1 day after induced luteolysis as indicator of terminal follicular growth could enable us to design robust time- schedule for artificial insemination to achieve maximum conception rates, however more studies are needed to establish cut-off values as it may vary among breeds.

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