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Hormonal and metabolic effects on antral follicle count in dairy cows

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Abstract: This study aimed to investigate the relationship between antral follicle count (AFC) and hormonal profiles as well as metabolic status in dairy cows. A total of 32 Holstein cows aged between 2 and 4 years and in their 60th day of lactation were included. Based on ultrasonographic evaluations, the cows were divided into two groups according to their AFC: low AFC (<24) and high AFC (≥24). Following ovulation synchronization, follicular development was monitored on the day of ovulation (Day 0) and subsequently on Days 3, 6, and 9. Blood samples collected on these days were analyzed for serum anti-Müllerian hormone (AMH), estradiol (E2), progesterone (P4), beta-hydroxybutyrate (BHBA), and cholesterol (CHOL) levels. The results showed no significant differences in AMH levels between the low and high AFC groups during the study period ($P > 0.05$). However, E2 levels on Day 9 were significantly higher in the low AFC group compared to the high AFC group ($P = 0.004$). Additionally, P4 levels were significantly higher in the low AFC group on Days 0, 6, and 9 ($P < 0.05$). In contrast, BHBA levels were significantly elevated in the high AFC group across all time points ($P < 0.05$). Cholesterol levels were consistently higher in the low AFC group throughout the study ($P < 0.05$). In conclusion, the findings suggest that AFC has a significant effect on hormonal profiles and energy metabolism in dairy cows. Particularly, the elevated steroid hormone levels observed in the low AFC group may reflect a compensatory mechanism to sustain reproductive function in conditions of limited follicular reserve.

Keywords: *Anti-Müllerian hormone, Antral follicle count, Dairy cows, Energy metabolism, Hormone profile, Metabolic parameters.*

Sütçü İneklerde Antral Folikül Sayısının Hormonal ve Metabolik Etkileri

Özet: Bu çalışma, süt ineklerinde antral folikül sayısı (AFC) ile hormonal profiller ve metabolik durum arasındaki ilişkiyi araştırmayı amaçlamıştır. Çalışmaya, 2 ile 4 yaşları arasında ve laktasyonun 60. gününde bulunan toplam 32 Holstein ineği dahil edilmiştir. Ultrasonografik değerlendirmelere göre inekler, AFC değerlerine göre düşük AFC (<24) ve yüksek AFC (≥24) olmak üzere iki gruba ayrılmıştır. Ovulasyon senkronizasyonunun ardından foliküler gelişim, ovulasyon günü (Gün 0) ve ardından 3., 6. ve 9. günlerde takip edilmiştir. Bu günlerde alınan kan örneklerinde serum anti-Müllerian hormonu (AMH), östradiol (E2), progesteron (P4), beta-hidroksibutirat (BHBA) ve kolesterol (CHOL) seviyeleri analiz edilmiştir. Sonuçlar, çalışma süresi boyunca düşük ve yüksek AFC grupları arasında AMH seviyelerinde anlamlı bir fark bulunmadığını göstermiştir ($P > 0.05$). Ancak, 9. günde E2 seviyeleri düşük AFC grubunda, yüksek AFC grubuna kıyasla anlamlı derecede daha yüksek saptanmıştır ($P = 0.004$). Buna ek olarak, 0., 6. ve 9. günlerde düşük AFC grubunda P4 seviyeleri anlamlı derecede daha yüksek bulunmuştur ($P < 0.05$). Buna karşılık, tüm zaman noktalarında yüksek AFC grubunda BHBA seviyeleri istatistiksel olarak anlamlı düzeyde daha yüksek tespit edilmiştir ($P < 0.05$). Kolesterol seviyeleri ise çalışma boyunca düşük AFC grubunda tutarlı bir şekilde daha yüksek bulunmuştur ($P < 0.05$). Sonuç olarak, elde edilen bulgular AFC'nin süt ineklerinde hormonal profiller ve enerji metabolizması üzerinde önemli bir etkisi olduğunu göstermektedir. Özellikle düşük AFC grubunda gözlemlenen artmış steroid hormon seviyeleri, sınırlı foliküler rezerv koşullarında üreme fonksiyonunun sürdürülebilmesi için bir telafi mekanizmasını yansıtıyor olabilir.

Anahtar Kelimeler: *Anti-Müllerian hormon, Antral folikül sayısı, Enerji metabolizması, Hormon profili, Metabolik parametreler, Süt inekleri.*

Introduction

Reproductive performance in dairy cows is a critical factor for enhancing economic efficiency in livestock production (Tohumcu and Tohumcu, 2024). It directly influences herd renewal rates and milk yield, while also playing a decisive role in reducing production costs and ensuring the sustainability of farm profitability. In this context, antral follicle count (AFC) has emerged as a significant biomarker for evaluating reproductive potential. AFC, which represents the number of follicles visible on ultrasonography, is widely utilized in fertility assessments due to its ability to reflect ovarian reserve (Guanga et al., 2022; Ireland et al., 2008; Moon et al., 2024).

The high repeatability of AFC and its substantial inter-individual variability enhance its value as a predictive tool for reproductive performance (Gobikrushanth et al., 2017; Koyama et al., 2018). Antral follicles form a pool of follicles capable of responding to gonadotropins and achieving ovulation. Consequently, AFC not only serves as an indicator of an individual's reproductive potential but also reflects metabolic status, with implications for both biological outcomes and economic efficiency (Morotti et al., 2022).

Hormones, which regulate various biological processes, are chemical compounds produced and secreted by multiple structures, including the ovaries. The developing follicles and other ovarian structures play a significant role in hormone production and secretion (Clark et al., 2022). Anti-Müllerian hormone (AMH), a glycoprotein secreted by granulosa cells, is a key indicator of follicular development (Juengel et al., 2021). Although the relationship between AMH levels and AFC has been explored in several studies (Guanga et al., 2022; Ireland et al., 2008; Moon et al., 2024), there remains limited information on the dynamic nature of this relationship and its variations across different stages of the estrous cycle. Steroid hormones, such as estradiol (E2) and progesterone (P4), are central to the regulation of the reproductive cycle (Bosolasco et al., 2021). The synthesis and metabolism of these hormones are closely tied to cholesterol metabolism (Patel et al., 2019). In addition, beta-hydroxybutyrate (BHBA) levels are considered an important marker of negative energy balance and metabolic stress (Ducháček et al., 2023).

This study was conducted under the hypothesis that AFC significantly affects hormone levels and metabolic status in dairy cows. The aim was to elucidate the relationships between AFC and key hormonal (AMH, E2, and P4) and metabolic parameters (BHBA and cholesterol), thereby contributing to a better understanding of the interplay between reproductive and metabolic processes in dairy cows.

Materials and Methods

Animals and Experimental Design: This study was conducted on 32 lactating Holstein cows housed at the Food and Livestock Application and Research Center. The cows were selected based on the following criteria: being 2 to 4 years old, at the 60th day of lactation, having a body condition score (BCS) between 2.75 and 3.50, having no history of reproductive or metabolic disorders, and displaying regular estrous cycles in the last two cycles. Following ultrasonographic evaluations, the cows were divided into two groups according to their antral follicle counts: the low AFC group (n=16, AFC<24) and the high AFC group (n=16, AFC≥24) (Sakaguchi et al., 2018). The study was approved by the Atatürk University Local Ethics Committee of Animal Experiments (Decision Number: 2021/91). All cows were housed under identical conditions and were fed a total mixed ration (TMR) formulated to meet NRC (2001) requirements. Throughout the study, ad libitum access to clean water was provided. Routine veterinary care was meticulously maintained, and all cows were deemed healthy based on pre-experiment clinical examinations. The cows were milked twice daily according to a standard milking protocol, and reproductive health records were systematically maintained.

Synchronization Protocol and Follicular Wave Monitoring: A standard 7-day ovulation synchronization (Ov-Synch) protocol, in combination with P4, was applied to all cows. The protocol included an initial GnRH injection on day -10, followed by PGF2α administration on day -3, and a second GnRH injection on day -1. During the Ov-Synch procedure, an intravaginal progesterone-releasing device (PRID® Delta, Ceva Sante Animale, Libourne, France) was inserted under aseptic conditions, and 10 mcg of buserelin acetate (Receptal®, MSD, Unterschleissheim, Germany) was administered intramuscularly. Seven days later, the PRID device was removed, and an intramuscular injection of 0.075 mg cloprostenol sodium (Estropur®, Bioveta, Ivanovice na Hane, Czech Republic) was given (Figure 1). Forty-eight hours after PRID removal, an additional 10 mcg of buserelin was administered intramuscularly (Hölper et al., 2023). Ovulation was monitored twice daily (at 09:00 and 21:00) using transrectal ultrasonography with a 7.5 MHz linear probe (Z60®, Mindray, Jiangsu, China). Cows in which ovulation was confirmed were included in the study, with the day of ovulation designated as day 0 (d 0). To monitor follicular development, further ultrasonographic examinations were performed on days 3, 6, and 9. during each examination, antral follicles with a diameter of ≥3 mm were identified, counted, and recorded.

Blood Sampling and Hormonal Analysis: Blood samples were collected from the coccygeal vein on the days specified in Figure 1. Samples were then centrifuged at 1200 x g at 4 °C for 10 minutes, and the sera were stored at -80 °C for subsequent analysis of anti-Müllerian hormone (AMH) (#EA0241BO, BT LAB), estrogen (E2) (#EA0093BO, BT LAB), progesterone (P4) (#EA0008BO, BT LAB), beta-hydroxybutyrate (BHBA) (#E0267BO, BT LAB), and cholesterol (CHOL) (#E2030BO, BT LAB). All serum measurements were performed using bovine ELISA kits according to the manufacturer's protocols (Bioassay Technology Laboratory).

Statistical analysis: Data normality was assessed with the Shapiro-Wilk test. A two-way repeated measures ANOVA

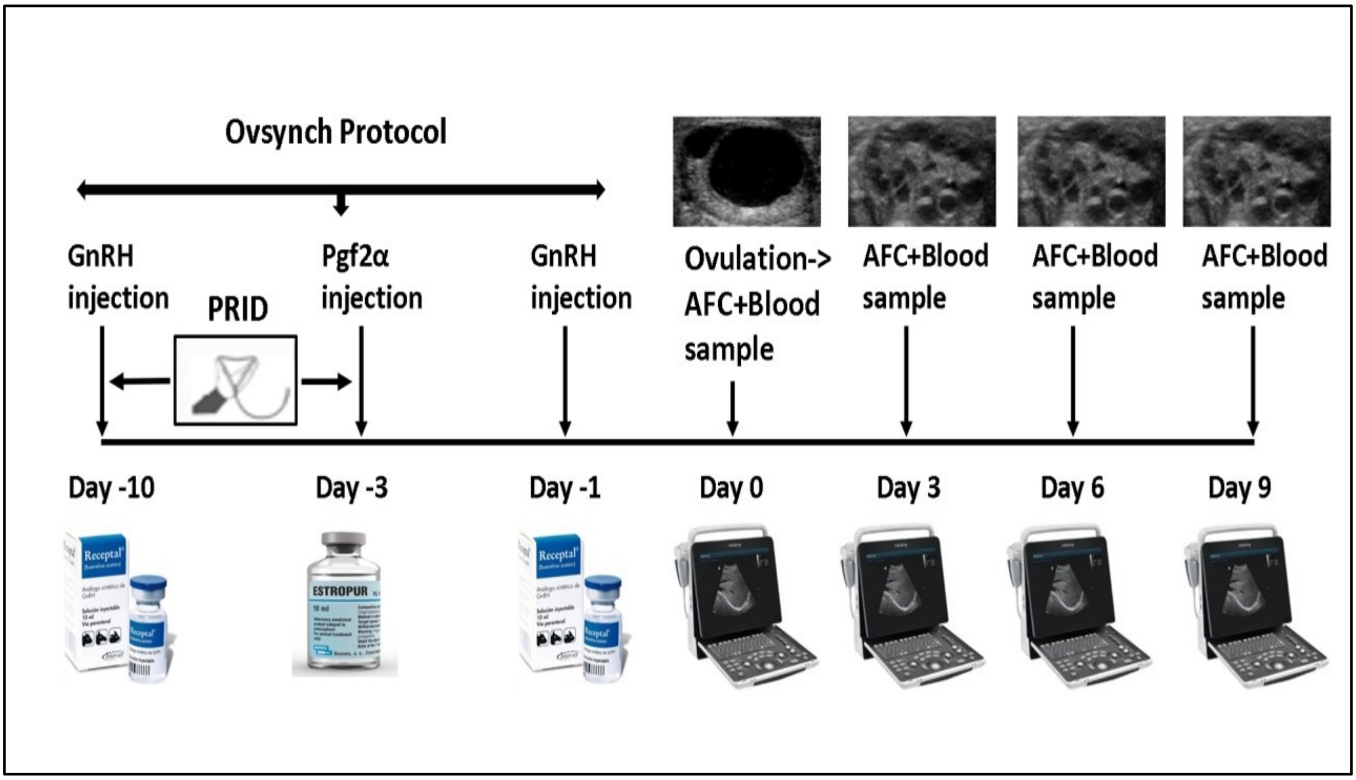

Figure 1. Synchronization Protocols, Blood Sampling, and Monitoring of Follicular Waves.

was employed to evaluate the effects of AFC group (low vs. high) and time (days 0, 3, 6, and 9) on hormone and metabolic parameters. Post-hoc comparisons were performed using Bonferroni correction. Pearson correlation analysis was used to examine the relationship between AFC and hormonal/metabolic profiles. All statistical analyses were performed using Medcalc version 20.2 (Medcalc Software Ltd., Ostend, Belgium) and SPSS version 25.0 (IBM Company, SPSS, IL, USA). A significance threshold of $P < 0.05$ was applied to all statistical tests (Table 1).

Table 1. Plasma anti-Müllerian hormone (AMH), estradiol (E2), progesterone (P4), beta-hydroxybutyrate (BHBA) and cholesterol (CHOL) measurement in low antral follicle count (AFC) and high AFC.

Hormones	Groups	Time-points				
		T_0	T_3	T_6	T_9	$P^†$
AMH (ng/L)	**Low AFC**	51 ± 22.8	42.5 ± 16.1	50 ± 20.4	46 ± 19.9	**0.245**
	High AFC	45.1 ± 10.7	50.4 ± 16	52.2 ± 23.8	55 ± 23.1	**0.277**
P^*		**0.356**	**0.177**	**0.728**	**0.236**	
E2 (ng/L)	**Low AFC**	146 ± 58.2	130 ± 57	118 ± 48.1	126 ± 21.1^a	**0.290**
	High AFC	113 ± 54.6	100 ± 36.4	99 ± 35.8	95.2 ± 34^b	**0.230**
P^*		**0.103**	**0.085**	**0.219**	**0.004**	
P4 (ng/mL)	**Low AFC**	15.7 ± 6.32^a	13.8 ± 6	12.1 ± 4.40^a	12.87 ± 3^a	**0.115**
	High AFC	9.80 ± 3.72^b	12 ± 5.82	9.27 ± 3.47^b	9.37 ± 4.12^b	**0.063**
P^*		**0.003**	**0.404**	**0.05**	**0.010**	
BHBA (nmol/mL)	**Low AFC**	186 ± 84^a	169 ± 66^a	190 ± 102^a	183 ± 67^a	**0.438**
	High AFC	268 ± 121^b	252 ± 85^b	259 ± 88^b	262 ± 90^b	**0.871**
P^*		**0.033**	**0.004**	**0.047**	**0.009**	
Chol (mg/dl)	**Low AFC**	156 ± 90^a	173 ± 100^a	155 ± 78.2^a	146 ± 68.2^a	**0.572**
	High AFC	89.5 ± 19.9^b	87.9 ± 22.9^b	85.8 ± 34.8^b	82.2 ± 24.4^b	**0.560**
P^*		**0.007**	**0.002**	**0.003**	**0.001**	

Data were expressed as mean ± standard deviation. Different letters indicated significant difference between groups at the same time ($p < 0.05$). P^* values showed significant difference between groups at the same time while $P^†$ values demonstrated significant difference between different time-point in same groups.

Results

Hormonal and Metabolic Parameters in Low versus High AFC Groups

AMH Profile

No significant differences were observed in AMH concentrations between low and high AFC groups throughout the study period (P > 0.05). In the low AFC group, mean (± SD) AMH concentrations at T0, T3, T6, and T9 were 51 ± 22.8, 42.5 ± 16.1, 50 ± 20.4, and 46 ± 19.9 ng/L, respectively. Corresponding values in the high AFC group were 45.1 ± 10.7, 50.4 ± 16.0, 52.2 ± 23.8, and 55 ± 23.1 ng/L. Temporal changes in AMH concentrations were not significant within either group (P = 0.245 for low AFC; P = 0.277 for high AFC) (Table 1).

Steroid Hormone Profiles

Estradiol

A significant difference in E2 concentrations between groups was observed only at T9, with the low AFC group showing higher concentrations compared to the high AFC group (126 ± 21.1 vs 95.2 ± 34.0 ng/L, respectively; P = 0.004). No significant differences were detected at other time points (P > 0.05). Temporal changes in E2 concentrations were not significant within either group (P = 0.290 for low AFC; P = 0.230 for high AFC) (Table 1).

Progesterone

The low AFC group exhibited significantly higher P4 concentrations compared to the high AFC group at T0 (15.7 ± 6.32 vs 9.80 ± 3.72 ng/mL; P = 0.003), T6 (12.1 ± 4.40 vs 9.27 ± 3.47 ng/mL; P = 0.050), and T9 (12.87 ± 3.00 vs 9.37 ± 4.12 ng/mL; P = 0.010). No significant difference was observed at T3 (P = 0.404). Temporal changes in P4 concentrations were not significant within either group (P = 0.115 for low AFC; P = 0.063 for high AFC) (Table 1).

Metabolic Parameters

Beta-hydroxybutyrate

BHBA concentrations were consistently higher in the high AFC group compared to the low AFC group across all time points: T0 (268 ± 121 vs 186 ± 84 nmol/mL; P = 0.033), T3 (252 ± 85 vs 169 ± 66 nmol/mL; P = 0.004), T6 (259 ± 88 vs 190 ± 102 nmol/mL; P = 0.047), and T9 (262 ± 90 vs 183 ± 67 nmol/mL; P = 0.009). No significant temporal changes were observed within either group (P = 0.438 for low AFC; P = 0.871 for high AFC) (Table 1).

Cholesterol

The low AFC group maintained significantly higher cholesterol concentrations compared to the high AFC group throughout the study period: T0 (156 ± 90 vs 89.5 ± 19.9 mg/dL; P = 0.007), T3 (173 ± 100 vs 87.9 ± 22.9 mg/dL; P = 0.002), T6 (155 ± 78.2 vs 85.8 ± 34.8 mg/dL; P = 0.003), and T9 (146 ± 68.2 vs 82.2 ± 24.4 mg/dL; P = 0.001). Temporal changes in cholesterol concentrations were not significant within either group (P = 0.572 for low AFC; P = 0.560 for high AFC) (Table 1).

Discussion and Conclusion

The lack of a significant difference in AMH concentrations between AFC groups was an unexpected finding, as AMH is widely regarded as a reliable marker of follicular reserve (Schwarzmann et al., 2023). This outcome contrasts with previous studies that have reported positive correlations between AFC and AMH levels (Baldrighi et al., 2014; Guanga et al., 2022; Ireland et al., 2008; Rico et al., 2009). However, our results align with research suggesting that changes in follicular development or hormone concentrations may be influenced by metabolic status (İleritürk & Kaynar, 2023; Rosa et al., 2021; Song et al., 2021).

Unlike findings from other studies (Modina et al., 2014; Sakaguchi et al., 2019), elevated E2 concentrations were observed in the low AFC group on day 9 (T9). Bonato et al. (2022) reported larger dominant follicle diameters in cows with low AFC, which could explain the increased E2 production (De los Reyes et al., 2006). The elevated E2 levels observed on T9 may therefore indicate distinct follicular dynamics in these cows. Similarly, the persistently high P4 levels observed in the low AFC group could be associated with differences in CL size (Bonato et al., 2022). While our findings partially parallel studies by Jimenez-Krassel et al. (2015) and Modina et al. (2014), which documented varying steroidogenic capacities among animals with differing AFC, they also diverge from studies linking low AFC to reduced P4 levels (Mossa et al., 2012; Mossa & Ireland, 2019; Sakaguchi et al., 2018). The elevated steroid hormone concentrations in cows with low AFC may represent a compensatory mechanism, reflecting increased steroidogenic activity per follicle to sustain reproductive function despite a reduced follicle count (Bonato et al., 2022; Mossa et al., 2012). Additionally, when coupled with more favorable metabolic profiles (lower BHBA levels), the higher P4 concentrations in low AFC cows suggest improved luteal function and potentially enhanced fertility, even with a diminished follicular reserve.

In dairy cows, circulating BHBA is a well-established biomarker of negative energy balance (NEB), which is known to impair follicular development (Gong et al., 2022; Missio et al., 2022). BHBA levels are generally considered normal when below 1 mmol/L (1000 nmol/mL) (Fiore et al., 2020). In vitro studies have demonstrated that direct injection of BHBA into follicles reduces follicle diameter and decreases E2 and P4 production by granulosa cells (Missio et al., 2022). Moreover, BHBA negatively impacts follicular development by reducing IGF-1 levels (Matoba et al., 2012). In our study, BHBA levels were within the normal range across all cows. However, the persistently higher BHBA levels in the high AFC group likely reflect a more pronounced energy deficit compared to the low AFC group. This observation could be attributed to the increased glucose utilization required for follicular development, as a higher number of antral follicles imposes a greater metabolic energy demand (Gamarra et al., 2015). Furthermore, the elevated cholesterol concentrations in the low AFC group, along with higher E2 and P4 levels compared to the high AFC group, are consistent with the positive association between cholesterol and steroid hormone synthesis. Previous studies have reported that embryos from high AFC cows contain less cholesterol than those from low AFC cows (Rosa et al., 2021). These findings suggest that the increased cholesterol levels observed in low

AFC cows may reflect differences in precursor availability for steroidogenesis or alterations in hepatic lipid metabolism (Anderson et al., 2015; Mathey et al., 2017).

In conclusion, AFC appears to have a significant impact on hormonal and metabolic processes in dairy cows. Notably, the high cholesterol and low BHBA levels observed in the low AFC group suggest that this group may possess a more favorable metabolic profile and improved luteal function. On the other hand, the increased follicular development in the high AFC group seems to elevate metabolic energy demands, potentially leading to higher BHBA levels. These findings highlight the potential of AFC as a critical biomarker for both reproductive performance and energy metabolism.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This study was approved by the Ataturk University Animal Experiments Local Ethics Committee (20.04.2021, 2021/91 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

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Writing the Article: VT, MC
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