**Original Research Article**

**Morphometric Analysis of Ovaries and Follicles and Their Relationship with Oocyte Quality in Embryo Donor Cows**

**ABSTRACT**

This study aimed to evaluate the morphological characteristics of ovaries, follicles, and corpora lutea in heifers, primiparous, and multiparous cows using ultrasound, and to assess the potential of ultrasound for follicle quality evaluation in embryo donor cow selection. Thirty cows, of which there were 10 heifers, 10 primiparous cows, and 10 multiparous cows, were examined using a 5 MHz transrectal ultrasound probe (Caresono Technology Co., Ltd, HD 9300, China). In ten reproductive-age cows (2.5 to 5.5 years), corpora lutea were observed in 7 right ovaries, follicles in 2, and no structures in 1. In ten heifers, corpora lutea were present in 4 right ovaries, with 2 also exhibiting follicles. Left ovaries in heifers showed corpora lutea in 5 and follicles in 3. In ten late-parity cows, corpora lutea were found in 6 right ovaries, and follicles in 5. Left ovaries of late-parity cows showed corpora lutea in 6, follicles in 6, corpora lutea and follicles in 3, and 1 non-functional ovary. Heifers exhibited statistically significantly smaller right ovary length and width (p < 0.05) compared to primiparous and multiparous cows. The examination also revealed smaller left ovaries in heifers compared to the other examined cows, though the difference was not statistically significant. Ultrasound alone cannot contribute to making definitive decisions regarding the selection of embryo donor cows. Apart from determining ovarian morphometric characteristics, it is necessary to assess hormone concentrations, particularly Anti-Müllerian hormone.

*Keywords****:*** *donor, ovary, follicle, corpus luteum*

**1. INTRODUCTION**

Reproduction is crucial in livestock production, and follicular evaluation provides a valuable method for assessing ovarian function, as follicles are the ovary's functional units, each containing an oocyte and associated somatic cells. Studies suggest a correlation between follicle size and oocyte quality; oocytes from larger follicles exhibit greater developmental potential for higher quality in vitro embryo production compared to those obtained from smaller follicles. Ovarian development is similar during embryogenesis; however, some studies prove functional differences between ovaries, in favor of the right ovary. Approximately 60% of ovulations occur from the right ovary, compared to 40% from the left. These differences are assumed to be related to the ovulatory response of each ovary, as well as pregnancy rates, and may be influenced by uterine conditions. Alternative examinations include the left ovary’s proximity to the rumen, potentially affecting it through temperature or pressure fluctuations and mechanical contractions.

This study aimed to measure the dimensions of the left and right ovaries and to identify specific ovarian structures across different age categories of cows.

**2. MATERIAL AND METHODS**

The study was conducted on a cattle milking farm owned by Nemanja Ilić in Živkovo, Leskovac municipality. The experiment included thirty heads of cattle of different ages or reproductive stages: 10 were heifers (never calved), 10 were mid-parity cows aged 2.5 to 6.5 years, with 1 to 3 previous calvings (primiparous), and 10 were late-parity cows aged 9 to 15 years, with 6 or more previous calvings (multiparous). Examinations of the reproductive tract, or ovaries as the primary target of this study, were performed via clinical examination, an ultrasound machine, and a 5 MHz transrectal probe (Caresono Technology Co., Ltd, HD 9300, China)

**2.1.** **Statistical Analysis**

The normality of the data distribution was assessed using the Shapiro-Wilk test. As the data were determined to be normally distributed (Shapiro-Wilk test, *P*>0.05), a one-way analysis of variance (ANOVA) was employed to compare the groups. Post-hoc comparisons were conducted using Tukey's test. Data are presented in tabular format. Statistical analyses were performed using GraphPad Prism version 7 (GraphPad, San Diego, CA, USA).

**3.** **RESULTS AND DISCUSSION**

***Table 1.*** *Ages in months by research groups*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Groups** | **n** | $$\overbar{x}$$ | **SD** | **X max** | **X min** | **Cv (%)** |
| **P** | 10 | 45.60 | 12.07 | 66.00 | 30.00 | 26.46 |
| **H** | 10 | 16.00 | 2.31 | 20.00 | 12.00 | 14.43 |
| **M** | 10 | 123.60 | 25.95 | 168.00 | 96.00 | 21.00 |

The M group had an average age of 123.60 ± 25.95 months, which was statistically significantly higher (*P*<0.001) compared to both the P (45.60±12.07) and H (16.00±2.31) groups, while the P (45.60±12.07) group also had a statistically significantly higher age than the H (16.00±2.31) group.



**Graph 1.** Length and width of the right ovary in heifers (A), primiparous cows (B), and multiparous cows (C). *Statistically significant differences are indicated by* *\* (p<0.05)*

The length of the right ovary was found to be significantly smaller in heifers (A) compared to both primiparous (B) and multiparous (C) cows (*P* <0.05). Specifically, the right ovary length in heifers was 29.74% smaller than in multiparous cows and 31.90% smaller than in primiparous cows. Similarly, the width of the right ovary in heifers was significantly smaller, by 26.63% compared to multiparous cows and by 30.77% compared to primiparous cows.



**Graph 2.** Length and width of the left ovary in heifers (A), primiparous cows (B), and multiparous cows (C).

Although no statistically significant differences were observed in the length of the left ovary among the groups, the left ovary length in heifers (A) was 24.54% smaller than in primiparous cows (B) and 21.30% smaller than in multiparous cows (C). The width of the left ovary in heifers was 9.20% smaller than in primiparous cows and 21.84% smaller than in multiparous cows.

***Table 2.*** *Structures on the right ovary in the test group*

|  |  |  |  |
| --- | --- | --- | --- |
| Parameter | P | H | M |
| n (%) | n (%) | n (%) |
| F | 3 (27.27) | 4 (33.33) | 6 (42.86) |
| CL | 7 (63.64) | 4 (33.33) | 7 (50.00) |
| Non-functional | 1 (9.09) | 3 (25.00) | 1 (7.14) |
| CLC | 0 (0.00) | 0 (0.00) | 0 (0.00) |
| FC | 0 (0.00) | 1 (8.33) | 0 (0.00) |

***Table 3.*** *Structures on the left ovary in the test groups*

|  |  |  |  |
| --- | --- | --- | --- |
| Parameter | P | H | M |
| n (%) | n (%) | n (%) |
| F | 4 (36.36) | 4 (33.33) | 6 (46.15) |
| CL | 5 (45.45) | 5 (41.67) | 5 (38.46) |
| Non-functional | 2 (18.18) | 3 (25.00) | 1 (7.69) |
| CLC | 0 (0.00) | 0 (0.00) | 1 (7.69) |
| FC | 0 (0.00) | 0 (0.00) | 0 (0.00) |

***Table 4****. Structures on the right ovary in the test groups*

|  |  |  |  |
| --- | --- | --- | --- |
| Parameter | P | H | M |
| n (%) | n (%) | n (%) |
| 2xCL | 0 (0.00) | 0 (0.00) | 1 (11.11) |
| 2xF | 1 (10.00) | 0 (0.00) | 1 (0) |
| F & CL | 0 (0.00) | 2 (20.00) | 0 (0.00) |
| CL | 7 (70.00) | 2 (20.00) | 1 (11.11) |
| F | 1 (10.00) | 2 (20.00) | 3 (33.33) |
| Non-functional | 1 (10.00) | 3 (30.00) | 3 (33.33) |
| Cyst | 0 (0.00) | 0 (0.00) | 1 (11.11) |
| F & CLC | 0 (0.00) | 0 (0.00) | 0 (0.00) |
| FC | 0 (0.00) | 1 (10.00) | 0 (0.00) |

***Table 5.*** *Structures on the left ovary in the test groups*

|  |  |  |  |
| --- | --- | --- | --- |
| Parameter | P | H | M |
| n (%) | n (%) | n (%) |
| 2xCL | 0 (0.00) | 0 (0.00) | 0 (0.00) |
| 2xF | 0 (0.00) | 1 (10.00) | 0 (0.00) |
| F & CL | 2 (20.00) | 1 (10.00) | 0 (0.00) |
| CL | 3 (30.00) | 4 (40.00) | 2 (20.00) |
| F | 3 (30.00) | 1 (10.00) | 3 (30.00) |
| Non-functional | 2 (20.00) | 3 (30.00) | 3 (30.00) |
| Cyst | 0 (0.00) | 0 (0.00) | 1 (10.00) |
| F & CLC | 0 (0.00) | 0 (0.00) | 0 (0.00) |
| FC | 0 (0.00) | 0 (0.00) | 1 (10.00) |

In this study, all 30 cows exhibited follicular activity; of these, 15 ovaries had a single follicle each, while the remaining four cows had a single follicle on both ovaries. Follicles were categorized into three size groups, the first measuring up to 3 mm, the second measuring 3–8 mm, and the third measuring over 8 mm. There were a total of nine ovaries classified as afunctional, including one case of bilateral afunctionality. Our study, comprising 30 cows in total, with different ages and number of calvings, found that the right ovary was larger than the left in 18 cows, which is 60% of the total number of cows, and the left ovary was larger than the right in the remaining 12 cases, or 40% of the total number of cows. A greater number of corpora lutea were found on the left ovary in 17 cases compared to 16 cases with corpora lutea on the right ovary, with three of the 33 corpora lutea exhibiting a cavum. The number of ovaries with follicles was in favor of the right ovary (14:13, compared to the left ovary). Ten ovaries were afunctional, showing no functional structures. Testis size was found to be a moderately to highly heritable trait [15] and is positively associated with fertility in bulls [13]. However, the relationship between ovarian size, ovarian reserve, and fertility in female cattle remains unclear [5].

Considering the results within individual categories, the total number of corpora lutea observed across both ovaries in ten heifers was 14 (9 on the right ovary and 5 on the left). This indicates previous ovulatory activity in these animals.

In cows with one or two previous calvings, a total of 12 corpora lutea were found: 7 on the right ovary and 5 on the left ovary. In cows with more than two calvings (7-10), the total number of corpora lutea was 9, with 4 on the right ovary and 5 on the left. This observation supports the established decline in reproductive potential in cattle with increasing age, particularly after the fifth year of life [6].

The estrous cycle in cattle typically involves two or three waves of antral follicles, each with at least one dominant follicle. Significant variations in the number of growing follicles during these waves (8-56 per wave) in young heifers can be used for reliable antral follicle phenotyping [4, 9]. Conversely, cattle with a low number of growing follicles during follicular waves often exhibit phenotypic characteristics associated with infertility [12]. A single measurement of serum anti-Müllerian hormone (AMH) concentration serves as a reliable biomarker for ovarian reserve size [10]. Maternal nutrition and diseases during pregnancy can contribute to the inherently large variation in follicle numbers during follicular waves and, consequently, the ovarian reserve of offspring. The number of antral follicles is positively correlated with ovarian size and the total number of follicles and oocytes in adult cattle ovaries. A study by [9] demonstrated that cows in the low antral follicle (AF) group had 60% smaller ovaries (3.05 ± 0.33 g vs. 7.11 ± 0.41 g) and a substantially lower total number of (healthy + atretic) follicles and oocytes (88,960 ± 27,515 vs. 829,185 ± 248,327) compared to the high AF group. Cows in the low AF group also exhibited approximately 80% fewer morphologically healthy follicles and oocytes (6,016 ± 1,685 vs. 29,059 ± 4,564) and approximately 45% fewer morphologically healthy follicles and oocytes per gram of ovary (2,110 ± 371 vs. 3,869 ± 535) compared to cows in the high AF group.

Furthermore, the low AF group had 80-90% fewer (p<0.05 to p<0.01) of each type of morphologically healthy follicle and oocyte (e.g., primordial, transient) compared to the high AF group. These findings indicate a dramatic reduction (approximately 80%) in ovarian reserve size in adult cows with low AF compared to those with high AF.

Adult cattle with low AF counts exhibit a poorer superovulatory response compared to those with high AF counts. The significant variation in AF observed in age-matched adult cattle directly influences their superovulatory response [9]. Studies involving standard commercial superovulation and artificial insemination (AI) procedures in heifers with high and low AF counts have estimated the number of inoculations and aspirated oocytes/embryos [9]. The results demonstrated that low AF animals had a consequently lower number of ovulated follicles, resulting in fewer corpora lutea (CL), fewer aspirated early embryos/unfertilized oocytes on day 7, and a reduced number of transferable embryos per cow compared to high AF animals [21]. This observation was the first to confirm that adult cattle with low AF counts, relative to those with high AF counts, had a reduced superovulatory response and produced significantly fewer high-quality embryos for embryo transfer, consistent with findings in older, less fertile cattle [20]. This finding, coupled with the observation that cows with low AF counts also had significantly smaller ovaries and a reduced number of morphologically healthy follicles and oocytes [4], provides initial, albeit indirect, evidence linking a low number of follicles and oocytes in adult cattle ovaries with suboptimal fertility, independent of age. Young adult cattle with low AF counts, compared to those with high AF counts, display the following characteristics: (1) significantly smaller ovaries; (2) significantly lower total number of morphologically healthy follicles and oocytes; (3) reduced superovulatory response and fewer transferable embryos; (4) chronically increased gonadotropin secretion, but lower circulating AMH and progesterone concentrations during the estrous cycle; (5) reduced endometrial thickness; and (6) higher cumulus cell markers indicative of reduced oocyte quality [17] and females [19] compared to younger animals.

A single serum anti-Müllerian hormone (AMH) concentration measurement serves as a valuable biomarker for ovarian reserve size [10]. Studies have shown that adult cattle with low AMH concentrations, and consequently a reduced number of morphologically healthy oocytes, require a greater number of AI procedures to achieve pregnancy [18]. A positive correlation exists between AF count and oocyte aspiration during oocyte pick-up (OPU) sessions, as well as the number of embryos produced. Females with high AF counts yield more embryos compared to those with medium and low AF counts. Therefore, AF count is a reliable marker for evaluating ovarian reserve and is positively correlated with parameters such as the number of viable oocytes, blastocysts, and conception rates after AI.

In this context, cows can be categorized as having low, medium, or high AF counts based on the number of antral follicles (3 mm in diameter) detected via ovarian ultrasound.

The results of [18] suggest that corpora lutea (CL)-free ovaries are a potential source of high-quality follicles. This study provides valuable insights that can serve as a foundation for producing high-quality oocytes that could potentially be used for in vitro cattle production. It has also confirmed that CL presence influences follicle growth and development, and cellular degeneration, negatively impacting oocyte development and embryo production. Conversely, CL-free ovaries yield quality follicles and cumulus-oocyte complexes. In contrast to these reports [18], our study found CL in all categories of cows: 12 CL were found on both ovaries in primiparous cows, 9 CL were found on both ovaries in heifers, and 12 CL were found on both ovaries in multiparous cows. It has been reported that CL, as an extracellular material within the ovary, undergoes growth, maintenance, and regression, potentially affecting the length, diameter and weight of the ovary containing it [11]. However, our study found no significant differences in ovarian length and diameter between ovaries with and without CL, partially aligning with findings from another study [2]. Conversely, a study involving cattle, goats, and buffaloes reported significantly greater ovarian weight, length, and diameter in ovaries with CL (p<0.05) [1]. In our experiment, a total of 33 CL were observed on ovaries. Out of the 30 ovaries, 24 contained a CL and among these, 21 ovaries exhibited greater length and width compared to ovaries without a CL.

A higher number of follicles, both 2-6 mm and > 6 mm in diameter, were observed in ovaries without CL compared to those with CL. This may be attributed to the absence of progesterone activity, as CLs secrete progesterone, which inhibits follicle development. Consequently, a lower number of follicles were found in CL-containing ovaries, consistent with findings reported by [2]. A strong correlation exists between follicle diameter and oocyte maturation. Follicles with diameters in the 4-6 mm range are considered ideal. Notably, 62% more follicles within the 2-6 mm size range were found in non-CL ovaries compared to CL ovaries. Previous research has confirmed that the presence of CL negatively impacts follicular growth, development, and cellular integrity [14].

As a result, the presence of a CL negatively impacts oocyte developmental competence and embryo production. In our study, we observed four instances where one ovary contained only a follicle, while the contralateral ovary contained both a follicle and a CL. In two of these cases, the follicle on the CL-containing ovary was smaller than the follicle on the ovary without CL. Conversely, in the other two cases, the follicle on the CL-containing ovary was larger.

The growing dominant follicle exhibits linear growth during the selection phase, and upon reaching approximately 9 mm in diameter, it inhibits the growth of subordinate follicles [8]. This dominant follicle is considered both morphologically and functionally dominant [16] and reaches a growth plateau after the second growth period [7]. Ovarian response during superovulation induction in follicle-dominant animals can be improved by removing the dominant follicle [3]. Therefore, the results of this study suggest that selecting potential donor cows based on the presence or absence of a dominant follicle can significantly enhance embryo yield. Dominant follicle detection can be accurately achieved through a single ultrasound examination, based on the number of small follicles (3-8 mm diameter) at the time of superovulation induction. Comparing these findings with our results, we observed that in 7 out of 10 (20 ovaries) reproductive-period or **primiparous cows (P),** follicular activity was observed on one or both ovaries, with follicle sizes ranging from 3 to 10 mm; in **heifers**, follicular activity, on one or both ovaries, was observed in 6 ovaries, with follicle sizes ranging from 6 to 15 mm; in 8 out of 10 **late-parity or multiparous cows (M),** follicular activity was observed on one or both ovaries, with follicle sizes ranging from 3 to 16 mm.

**4. CONCLUSION**

Based on our experimental results, ultrasound examination of the ovaries alone, focusing on the presence of ovarian structures, corpora lutea, and cysts of different natures, is insufficient for accurately selecting embryo donor candidates. Comprehensive donor selection requires supplementary hormonal assays, including anti-Müllerian hormone (AMH) analysis, and immunohistochemical testing, in addition to ultrasound morphometric analysis.

Previous findings indicate that the presence of a corpus luteum significantly influences follicular dynamics, with a lower number of follicles observed in CL-containing ovaries. This supports previous research suggesting that progesterone secretion from the CL suppresses follicular development. Additionally, the observed differences in follicle sizes between CL-containing and CL-free ovaries highlight the complexity of ovarian function and its impact on reproductive potential. Given the strong correlation between antral follicle count and ovarian reserve, our study underscores the importance of integrating ovarian ultrasonography with hormonal and molecular markers for a more precise assessment of fertility potential in donor cows. These insights contribute to refining selection criteria for embryo donors, ultimately improving reproductive efficiency and superovulation outcomes in cattle.

Future research should focus on longitudinal monitoring of follicular development dynamics and ovarian functionality in different categories of cows to establish a more precise correlation between ovarian size, antral follicle count, and fertility. Special attention should be given to the influence of age, nutritional status, and endocrinological parameters on ovarian reserve size and superovulation success.

Further investigation is required to assess the impact of the presence and morphological characteristics of the corpus luteum on the follicle and oocyte quality, given the findings that ovaries without a corpus luteum contain a higher number of optimally sized follicles. Understanding this relationship could contribute to optimizing superovulation protocols and enhancing the efficiency of reproductive biotechnologies in cattle.

**Ethical approval**

All authors hereby declare that "Principles of laboratory animal care" were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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