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Reproductive System Evaluation in Anestrus Buffaloes by Ultrasound and Antioxidants Profile in Basrah Governorate, Iraq

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ABSTRACT

The research study aimed to explore the reasons for buffaloes experiencing anestrus during their breeding season in Basrah governorate, Iraq. The research included a sample of 52 buffaloes suffering from anestrus and another five in the estrus phase. The reproductive system of these animals was examined to evaluate the causes of anestrus using trans-rectal ultrasonography. Antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-px), and the level of malondialdehyde (MDA) were measured in the blood of anestrus buffaloes and compared with those of normal cyclic buffaloes. Ultrasonographical results revealed that the infertile buffaloes suffered from ovarian inactivity, anovulatory follicles, persistent corpus luteum, and luteal cysts at rates of 34.16%, 21.15%, 17.30%, and 13.46%, respectively. The research found that 13.46% of buffaloes with normal estrous cycles experienced silent estrus. The results also showed a significant decrease (P<0.05) in CAT, SOD, and GSH-px in anestrus buffaloes compared with regularly cyclic buffaloes and a significant elevation (P<0.05) in the MDA level in anestrus buffaloes compared with the control group. The study concluded that the animals were under oxidative stress due to heat stress and a poor diet in highly productive buffaloes, which plays an important role in anestrus and infertility.

Introduction

Buffalo reproduction faces multiple genetic issues, resulting in low breeding success because its fertility rate remains below the required threshold (Perera, 2008). Multiple factors, including delayed puberty, seasonal breeding patterns, prolonged postpartum anestrus and intercalving interval, and insufficient signs of estrus, lead to decreased buffalo productivity (Drost, 2007). The global buffalo population declined sharply over the past 10 years, leading to the swamp buffalo being classified as an endangered species (Saikhun et al., 2002; Suteevun et al., 2006).

Anestrus in buffaloes constitutes the main reproductive problem that affects their ability to breed. Functional infertility in buffaloes represents the primary reason for buffalo infertility since this condition appears in more than

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50-60% of all infertility cases (Abdulkareem et al., 2021; Sah and Nakao, 2010). Repeat breeding in buffaloes is another factor that minimizes fertility during the breeding season (Saraswat and Purohit, 2016). The condition affected 29% of buffaloes, resulting in significant economic losses for buffalo farmers due to reduced reproductive performance and milk production (Kumar et al., 2013). The main factors affecting anestrus incidence in buffaloes, including management circumstances, environment, body condition score, nutritional deficiency, milk production, calving season, and genetics, contribute to anestrus incidence (Kumar et al., 2013).

The precise determination of the causes of anestrus in buffaloes must be established as the primary requirement for selecting effective treatments that restore normal estrus and reproductive system function (Kumar et al., 2019). The standard methods used to detect anestrus in buffalo through rectal palpation and clinical examination need specialized knowledge and precise skills to discover the primary reasons for anestrus; however, more precise diagnostic methods and treatment techniques are necessary (Rahman et al., 2012).

The most common method for diagnosing reproductive system disorders uses ultrasonography as its primary diagnostic tool (Jyoti et al., 2019). Sonography uses high-frequency sound waves to create images of internal organs, allowing the identification of abnormal tissues (Hayward, 2012), and enables the creation of images of body structures and provides reliable diagnostic information that is needed to identify and treat a wide range of medical conditions (Jyoti et al., 2019).

Previous studies have shown that reproductive performance is highly affected by stress factors (Khodaei-Motlagh et al., 2011; Dobson et al., 2020; Alsalim et al., 2023). The production of reactive oxygen species (ROS) depends on stressors, including heat stress, malnutrition, and diseases (Chauhan et al., 2021). The reproductive ability of animals is affected by ROS, which disrupts the biological processes of folliculogenesis and steroidogenesis, which are vital for maintaining normal estrous cycles during designated postpartum periods (Ghosh et al., 2015; Khan et al., 2020). Cells use both enzymatic and non-enzymatic antioxidant defense mechanisms to defend themselves against free radical damage by converting ROS into harmless substances, which protect their vital functions (Khan et al., 2020). Research has established a direct connection between enzymatic antioxidant levels and the likelihood of retained placenta in cows (Khudhair et al., 2021), buffaloes (Atallah and Moustafa, 2006), and females with polycystic ovaries (Papalou et al., 2016), as well as infertility in men (Tvrdá et al., 2011). The female reproductive system relies on various enzymatic antioxidants, including CAT, SOD, and GSH-px (Khan et al., 2020).

The purpose of this study was to focus on the ultrasonographical evaluation of the reproductive system and to evaluate the activity of enzymatic antioxidants in postpartum anestrus buffaloes to determine the main causes and features of each case of postpartum anestrus in buffaloes.

Materials and methods

Animal study

A total of 52 local buffaloes (aged between five and nine years) from private farms in the south of Iraq (Basrah and Thi-Qar governorates) were used in this study, which had suffered from anestrus without any observation of estrus signs after parturition (more than 4 months from parturition) during the breeding season (October to February). Another five buffaloes with a normal estrous cycle in the estrus phase were used as controls in this study.

Ultrasound examination

Ultrasonography was conducted through the application of a multi-frequency linear rectal probe (5.0 MHz) (Sofi and Singh 2018). The transducer face was lubricated with a proper coupling medium (Ultra Sonic Gel ® for medical use) and then progressed cranially along the rectum. The reproductive tract of each anestrus buffalo was examined (cervix, horns of the uterus, and ovaries). Uterine diameter, shape, and layers, as well as its content, such as fluid and pus, were examined. The ovary was also evaluated in postpartum anestrus buffalo, and its structures were determined.

Blood sample

Blood samples were collected from the jugular veins of anestrus buffaloes and buffaloes experiencing normal estrus cycles. Serum was extracted from blood samples by centrifuging them for 20 minutes at 3000 rpm. After labeling, it was stored at −20 °C until the research parameters were analyzed.

Antioxidants analysis

GSH-Px activity was measured at 412 nm at 37°C using a specific kit (Sza kits, Germany) according to Flohe and Gunzler (1984). The Aebi (1984) method was used to determine serum CAT activity, which was expressed as micromoles per milliliter (µM/ml). The specialized kit (Sza kits, Germany) was used to measure SOD activity in serum at 480 nm, with immediate testing at 37 °C, as described by Flohe and Gunzler (1984). According to the formula of Beuge and Aust (1978), the Malondialdehyde (MDA) level was measured at 25 °C and 532 nm. The APEL CO Ltd Japan PD 303 UV spectrophotometer was used for all analyses.

Statistical analysis

Utilizing the independent samples t-test in the SPSS software program, antioxidant activity and MDA were compared between anestrus and normal buffaloes (Version 21, IBM, USA). The data were displayed as mean ± SEM, with statistical significance defined as p values < 0.05.

Results

Ultrasonographic evaluation of the reproductive system in anestrus buffaloes revealed numerous vital findings (Table 1). The study showed that 34.62% of the study animals had inactive ovaries, characterized by isoechoic, smooth, gray ovaries with a high-echoic border and the absence of follicles (Figure 1, A). In contrast, the uterine horns revealed smooth, gray, hyper-echogenic, and indistinct border rings in transverse section (Figure 1, B). Moreover, 21.15% of anestrus animals had anovulatory follicles, which showed isoechoic functional gray ovaries with a high-echo border and small, irregular follicles (Figure 2, A). At the same time, the uterine horns became hyper-echogenic, with indistinct borders and a small, irregular lumen.

Table 1: The main causes for anestrus buffaloes diagnosed by ultrasound

Diagnosed causes	No. and percentage of cases (%)
Inactive ovaries	18 (34.62)
Anovulatory follicles	11 (21.15)
Persistent corpus luteum	9 (17.31)
Luteal cyst	7 (13.46)
Silent estrus	7 (13.46)
Total	52

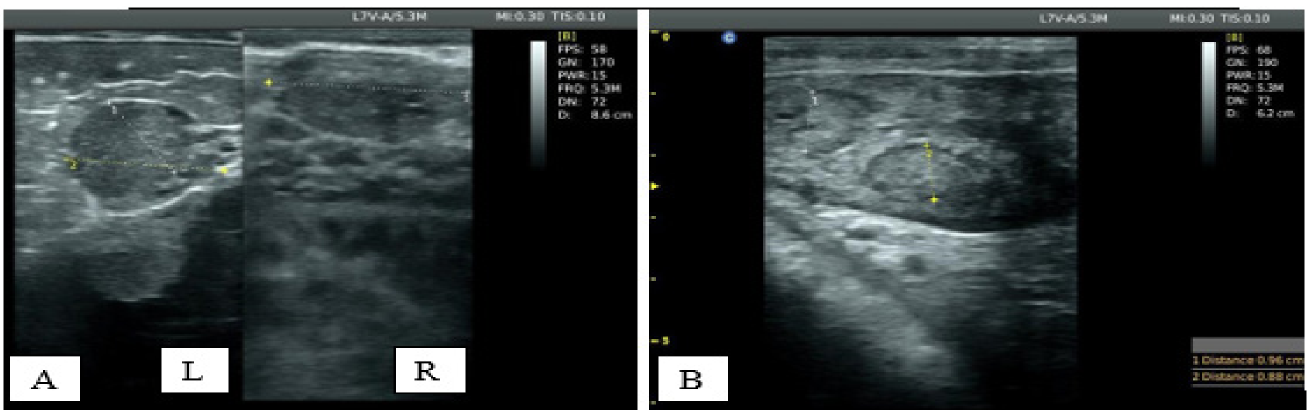

Figure 1: The ultrasound image displays inactive ovaries of anestrus buffaloes, shown in A. Ovaries (left (L) and right (R)) are isoechoic, smooth, and gray, with hyperechoic borders and absent follicles. B- Uterine horns with smooth gray hyperechogenic and unclear border rings in transverse section by using a 5 MHz trans-rectal transducer.

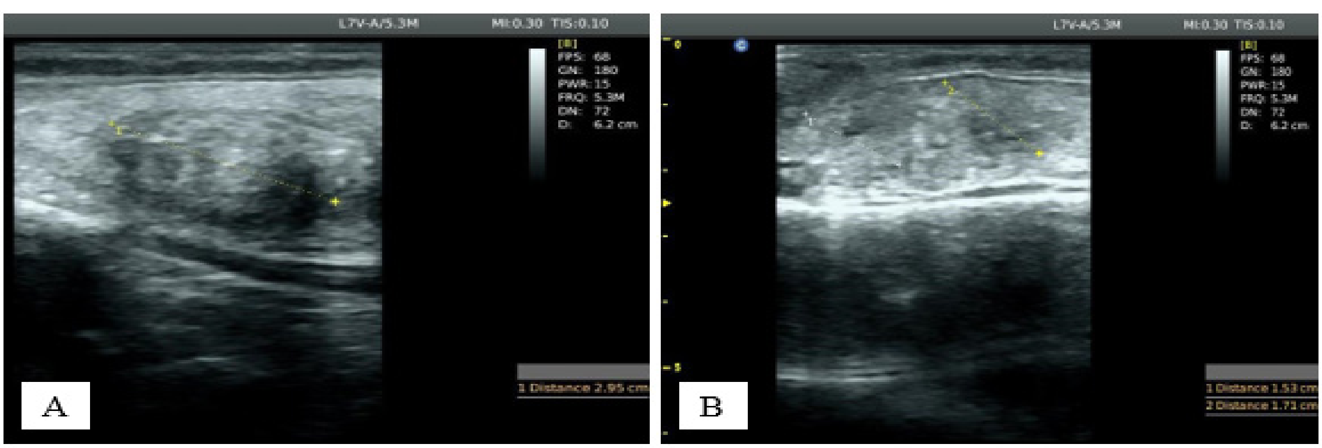

Figure 2: The ultrasound image displays an ovulatory follicle in an anestrous buffalo, shown in A. Ovaries showed isoechoic, smooth, gray ovaries with hyperechoic borders and small anovulatory follicles. B- Uterine horns with smooth gray hyperechogenic and unclear borders, with an unclear small lumen in transverse section by using a 5 MHz trans-rectal transducer.

The study found that 17.31% of anestrus cases maintain a persistent corpus luteum, which presents as dense theca tissue that shows greater echogenicity than ovarian tissue with a small central hypoechogenic vacuole (Figure 3, A and Figure 4, A). The uterus displayed hyperechogenic horns, a distinct gray ring, and hyperechogenic white secretion in its transverse section (Figure 3 B). The other cases displayed pyometra, which presented as hypoechoic inflammatory fluid inside the uterus with an increased thickness of the uterine wall (Figure 4, B).

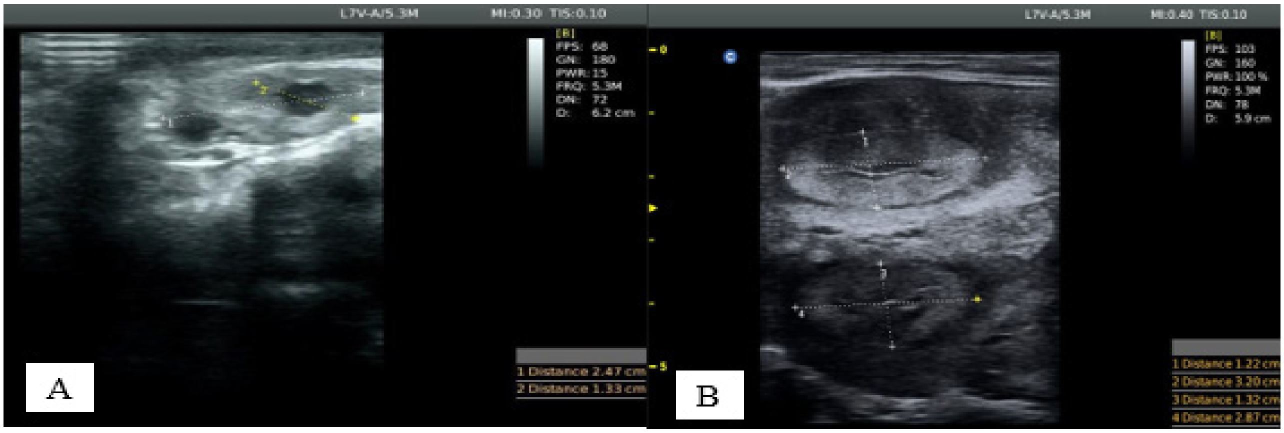

Figure 3: The ultrasound image shows a persistent corpus luteum, which exists in anestrus buffaloes, shown in A- The ovary contains dense theca tissue, which produces greater echogenicity than the normal ovarian echogenicity. B- The uterine horns show high echogenicity with a clear gray ring and hyperechoic white secretion that appears in transverse section by using a 5 MHz trans-rectal transducer.

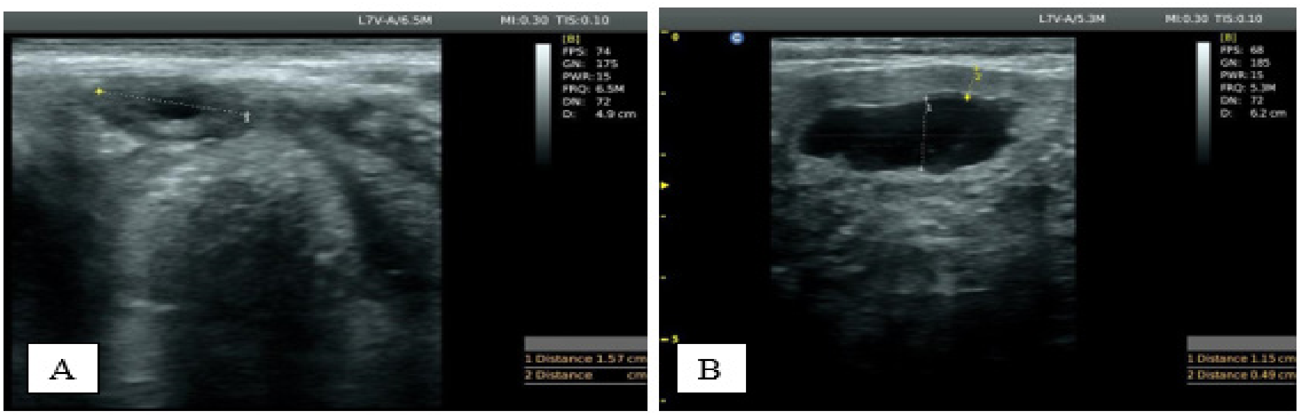

Figure 4: The ultrasound image shows a persistent corpus luteum accompanied by pyometra in anestrus buffaloes, shown in A- ovary with dense theca tissue more echoic than ovarian echogenicity, B- Uterine horns with thick and hypoechoic muco-inflammatory fluid, with thickened uterine lining by using a 5 MHz trans-rectal transducer.

The research found that 13.46% of anestrus buffaloes showed protruded luteal cysts, which presented as dense theca tissue with greater echogenicity than ovarian tissue and occupied most of the ovarian area (Figure 5, A). The uterine horns showed reduced echogenicity, which created a distinct gray ring and hyperechogenic white secretion that

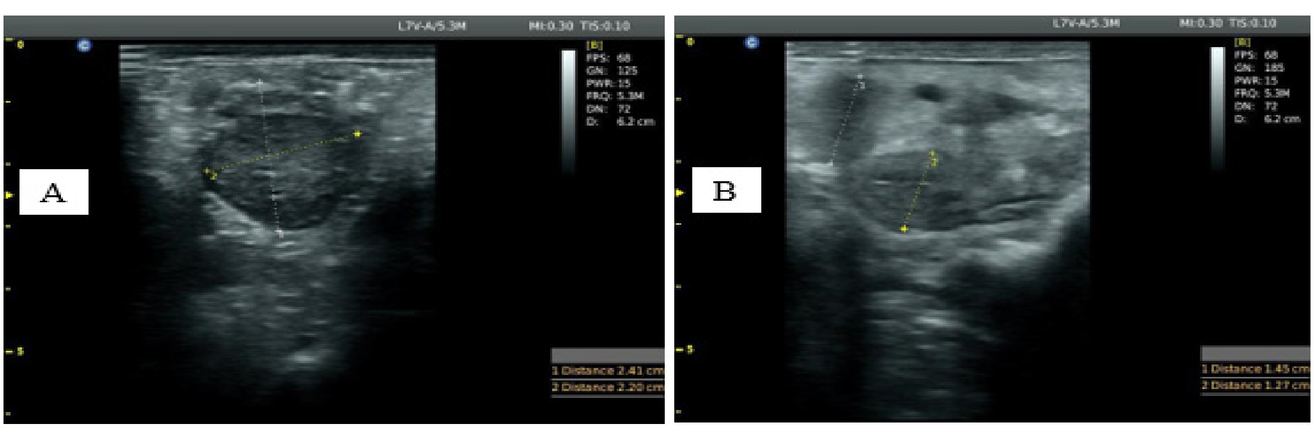

Figure 5: Ultrasound image of a luteal cyst in anestrus buffaloes. A- Ovaries with dense theca tissue protruded outside and are more echoic than the ovary. B- The uterine horns show hypoechogenicity with a clear gray ring and white secretion that appears in transverse section by using a 5 MHz trans-rectal transducer.

The research found that 13.46% of anestrus cases presented as silent estrus, and the ultrasound device demonstrated its diagnostic ability in these cases. The ultrasound images showed normal ovarian structures, including a mature follicle (Figure 6, A). The transverse section of the uterine horns displayed gray and clear echogenic uterine layers with anechoic dark fluid in the uterine lumen (Figure 6, B).

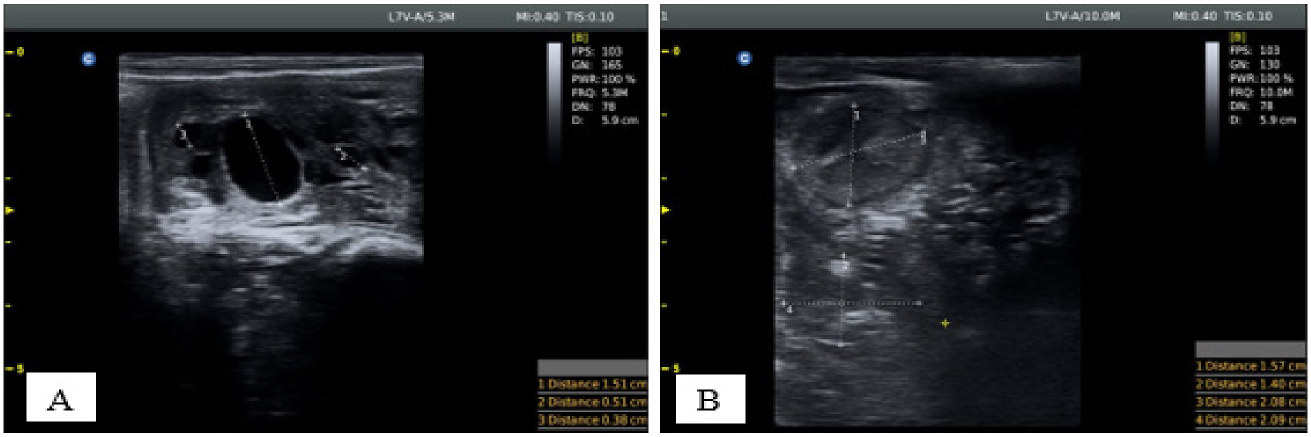

Figure 6: Ultrasound images demonstrate the presence of silent estrus in buffaloes. A- Ovaries show normal structural development with mature follicle formation. B- Uterine horns display gray and clear echogenicity with anechoic dark fluid in the uterine lumen that appears in transverse section by using a 5 MHz trans-rectal transducer.

Table 2 displays the antioxidant enzyme activity results found in buffaloes who experience infertility. The catalase enzyme (CAT) activity demonstrated a substantial drop ($P<0.05$) when researchers examined anestrus buffaloes against their control counterparts. The superoxide dismutase (SOD) activity reached its lowest significant value ($P<0.05$) in anestrus buffaloes who showed abnormal estrus behavior. The anestrus animals displayed a significant decrease ($P<0.05$) in glutathione peroxidase (GSH-Px) activity when compared to animals who exhibited a normal estrus cycle.

In the current study, enzymatic antioxidant activity in anestrus buffaloes showed significant decreases ($P<0.05$) in CAT, SOD, and GSH-Px activity compared with buffaloes with a normal estrus cycle (Table 2). Furthermore, stress index levels were evaluated using MDA levels, which revealed a significant increase ($P<0.05$) in their levels in anestrus buffaloes compared with those of buffaloes in normal estrus cycles.

Table 2: Antioxidant enzyme activity and MDA level in anestrus buffaloes compared to buffaloes with normal estrus cycle

Antioxidant enzyme	Anestrus buffaloes	Normal buffaloes
Catalase (µM/ml)	5.43±2.77 a	11.84±3.97 b
superoxide dismutase (µM/ml)	4.34±1.63 a	8.19±2.24 b
Glutathione peroxidase (µM/ml)	64.25±17.55 a	154.10±22.39 b
MDA (µM//L)	83.64±11.41 a	41.72±6.20 b

The data show results through the combination of mean value and standard error measurement. The use of different small letters in each row shows significant differences that reach a statistical level of $p<0.05$.

Discussion

Generally, reproduction in buffaloes is affected by many factors that contribute to reduced reproductive efficiency, such as anestrus, silent estrus, malnutrition, heat stress, infectious diseases, hormonal imbalance, genetic factors, and ovarian problems (Mopuri, 2024). Our study focused on the ultrasonographic evaluation of the reproductive system in anestrus buffaloes to determine the primary causes and identify the key features in each case.

In our study, up to 35 percent of the causes of infertility in buffaloes were attributed to inactive ovaries, in agreement with (Kumar et al., 2013). The main reasons for ovarian inactivity are nutritional deficiencies and inadequate pasture quantity and quality (Sah and Nakao, 2010; Vijayalakshmy et al., 2020), followed by a decline in gonadotropin-releasing hormone. Additionally, stress factors and heat stress are significant factors contributing to ovarian inactivity in buffaloes. Heat stress in buffaloes is considered a major cause of physiological and behavioral changes that interfere with their productivity and reproductive performance (Choudhary and Sirohi, 2019). Reducing heat stress in buffaloes is usually associated with improved milk production, reproductive performance, and endocrine status (Choudhary and Sirohi, 2019).

Our results revealed that anovulated follicles accounted for 21% of the causes, in agreement with (Awasthi et al., 2007). Ultrasound showed smooth, gray ovaries with a high-echogenic border and small anovulatory follicles, even after one week of monitoring. Anovulatory follicles are common in buffaloes, which exhibit a regular follicular wave pattern and follicular growth followed by regression and emergence of a new follicular wave a few days later (Manik et al., 2022).

The ovaries of such animals are small in dimensions because they lack both corpus luteum and ovulatory-size follicles. The follicle requires an LH pulse frequency to reach its growth stage after the follicular wave begins (Terzano, 2005), whereas inadequate LH levels result in anovulatory follicles.

The study results demonstrated that 17% of cases showed persistent corpus luteum, consistent with the findings of (Barkawi et al., 2009). The ultrasound shows a dense tissue structure that appears more echogenic than ovarian tissue and contains a small central echogenic vacuole. Most persistent corpus luteum cases were accompanied by uterine disease, especially pyometra and uterine infection, which agrees with (Sheldon et al., 2008). Uterine infection and diseases usually play a key role in inhibiting prostaglandin release and preventing luteolysis (Sheldon et al., 2008). A corpus luteum that remains active beyond its normal functional period continues to generate progesterone. The hormone progesterone reduces GnRH and LH production through its effects on the hypothalamus-pituitary axis, leading to the animal's temporary infertility period (Wiltbank et al., 2002).

The research demonstrated that luteal cysts were a cause of anestrus in buffaloes, accounting for about 13.5% of anestrus cases, according to the findings of (Kumer et al., 2014). The ultrasound examination of a luteal cyst showed dense theca tissue, which produced more echogenicity than the ovary and covered most of the ovarian area. Most cases of luteal cysts occurred in dairy buffaloes that showed high milk production but suffered from nutritional deficiencies (Amin et al., 2025). The combination of environmental stress and metabolic diseases that cause a negative energy balance in high-yielding dairy buffaloes leads to a decrease in gonadotropin-releasing hormone levels and an increased risk of luteal cyst development (Singh et al., 2000). The elevated prolactin levels in high-yielding animals lead to a reduction in gonadotropin-releasing hormone secretion, which results in decreased pituitary production of gonadotropins (Kumar, 2014).

The study results showed that 13% of the studied cases had normal estrus cycles without signs of estrus. Subestrus or silent estrus is defined as a condition where regular estrus cycles occur without the detection of visible signs of estrus despite the normal process of follicular development and ovulation (Kumer et al., 2021). Silent estrus is common in heifers after the post-pubertal period and during the early postpartum stage in high-yielding dairy animals (Kumer et al., 2021). This condition is usually reported in dairy buffaloes during the summer months and is a reason for longer calving intervals (Dash et al., 2016). The estrogen concentration in high-yielding dairy cows determines their behavioral estrus signs, which remain at low levels (Kumer et al., 2021). The body experiences decreased estrogen levels due to high metabolic activity, which accelerates estrogen elimination via metabolic processes (Singh et al., 2000), or suboptimal follicular development, in which mature follicles fail to produce enough estradiol (Terzano, 2005). In some cases, a higher threshold of estrogen in the central nervous system is required to display estrus symptoms as a heritable cause (Kumer et al., 2014). The combination of heat stress, nutritional deficiencies, overweight, foot lesions, aging, and the failure of estrus detection plays a major role in subestrus conditions (Kumer et al., 2021).

The current study examined the activity of antioxidant enzymes (CAT, SOD, and GSH_Px) in anestrus buffaloes and regular cyclic buffaloes. The study detected a significant reduction in these enzymes in anestrus buffaloes compared with their counterparts with normal cyclic behavior. The findings agree with those of (Ghosh et al., 2015), who documented similar evidence in anestrus buffaloes. Cows and ewes showed a similar pattern of decrease in their enzymatic antioxidants during anestrus (Alsalim et al., 2023; Duan et al., 2025). Antioxidant enzymes work to convert ROS together with their resulting substances into less harmful compounds while they protect cells from oxidative stress damage, which can result in cell damage (Bratovcic 2020). Previous studies found that human and animal diseases arise from insufficient antioxidant enzyme activity (Ozougwu, 2026; Bratovcic 2020). The reduction in enzymatic and non-enzymatic antioxidants permits ROS to interfere with cells and organs, resulting in oxidation and various medical conditions (Ozougwu, 2026; Bratovcic, 2020). Our study showed that anestrus buffaloes experienced oxidative stress because they endured stressors that include heat stress, abnormal puerperium, uterine infection, and their restricted dietary intake. Previous studies indicate that increased oxidative stress can directly or indirectly affect the production of reproductive hormones (Chainy and Sahoo, 2020) or the processes of folliculogenesis in females and spermatogenesis in males (Aitken, 2020). Increased oxidative stress negatively impacts the production of reproductive hormones in cows and can lead to postpartum anestrus (Hameed and Alsalim, 2023).

Our study detected oxidative stress in animals by measuring MDA levels, which showed higher levels in anestrus buffaloes than in their normal cyclic counterparts. This result agrees with (Perumal et al., 2021), which documented a sharp increase in MDA levels in anestrus water buffaloes. A similar finding in Cholistani cattle showed elevated MDA levels during both anestrus and repeat breeding (Ali et al., 2014). MDA serves as both a measurement of lipid peroxidation and an indicator of oxidative stress in tissue systems (Yekti et al., 2018).

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