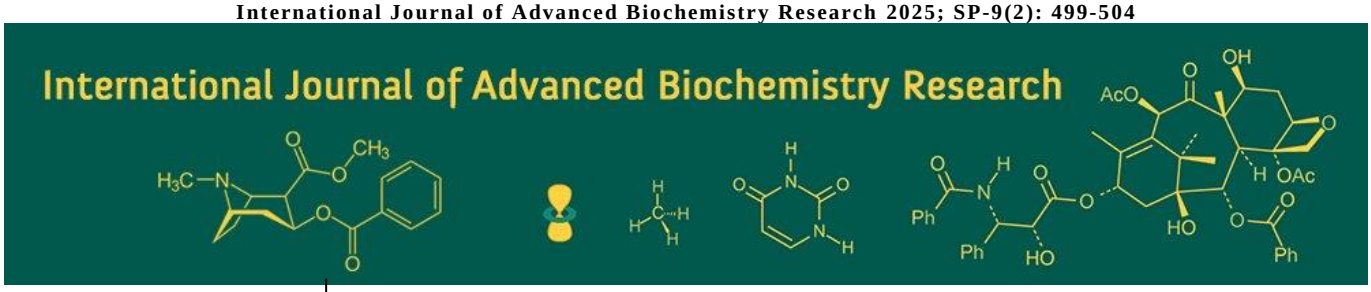

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Metabolic profiling of serum and follicular fluid across age and breeding seasons in ovine species

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Abstract
Metabolic processes in ruminants, including sheep, differ from those in monogastric animals due to their unique digestive system, which relies on microbial fermentation to produce volatile fatty acids (VFAs) as primary energy sources. Hepatic gluconeogenesis serves as the principal mechanism for glucose production in sheep, including during fetal development. The present study investigated the biochemical composition of serum and follicular fluid in ewes across different age groups (<1 year and >1 year) during breeding and non-breeding seasons to elucidate metabolic variations associated with reproductive status and environmental conditions. A total of 16 ewes were selected from a civil slaughterhouse in Bangalore, India, and categorized based on seasonality and age. Serum and follicular fluid samples were analyzed for biochemical metabolites, electrolytes, and enzymatic markers using commercially available diagnostic kits. The results indicated significant differences (p<0.05) in the concentrations of several biochemical constituents between serum and follicular fluid across age groups and seasons. Protein and albumin levels were higher in serum, while HDL, cholesterol, and triglycerides exhibited elevated concentrations in follicular fluid. Urea concentrations were significantly higher in follicular fluid, suggesting active transport or local synthesis. Seasonal variations influenced metabolic markers, with pregnant ewes during the breeding season displaying lower serum glucose and elevated β-hydroxybutyrate (BHBA) levels, indicative of a negative energy balance. Additionally, increased aspartate aminotransferase (AST) activity suggested hepatic stress in pregnant ewes, exacerbated by environmental factors such as winter cold stress. These findings provide critical insights into the metabolic regulation of serum and follicular fluid in ewes, contributing to a better understanding of ovarian function and reproductive physiology. The study highlights the impact of seasonality and pregnancy on metabolic homeostasis, emphasizing the importance of nutritional and environmental management in improving reproductive efficiency in sheep.

Keywords: Biochemical profiling, serum, follicular fluid, sheep, seasonality, reproductive physiology, metabolic stress

Introduction
Metabolic processes in ruminants, including sheep, differ significantly from those in non-ruminants due to the unique nature of their digestive system. Unlike monogastric animals, where ingested carbohydrates are primarily absorbed as glucose, ruminants metabolize dietary carbohydrates into short-chain volatile fatty acids (VFAs) through microbial fermentation in the rumen. Consequently, hepatic gluconeogenesis becomes the primary source of circulating glucose in sheep and other ruminants (Wang *et al*., 2012) [22]. Notably, gluconeogenesis is active even in the fetal sheep liver, ensuring a continuous supply of glucose during gestation (Thorn *et al*., 2012) [20]. To assess the metabolic status of ruminants, including potential disturbances, metabolic profiling of blood serum is essential. This profile includes biochemical markers and serum minerals, which serve as indicators of various physiological and pathological conditions. During pregnancy, ewes experience an increased energy demand to support fetal growth. However, this heightened demand is not always met by a proportional increase in endogenous glucose production, leading to a state of relative energy deficiency (Raoofi *et al*., 2013) [15]. This energy deficit results in the mobilization of fatty acids from adipose tissue, which are subsequently metabolized in the liver, leading to an increase in ketone body production, particularly β-hydroxybutyrate (BHBA) (Moallem *et al*., 2012) [12].

If this imbalance persists, it can result in pregnancy toxemia, a critical metabolic disorder characterized by neurological symptoms due to insufficient glucose availability for the brain (Radostits *et al*., 2006; Duehlmeier *et al*., 2013) [14, 3].
Several intrinsic and extrinsic factors contribute to the development of a negative energy balance (NEB), including feed intake, hormonal status, age, reproductive stage, environmental stressors, and multiple pregnancies (Cal-Pereyra *et al*., 2015; Regnault *et al*., 2004) [2, 16]. Seasonal variations further influence metabolic activity in sheep. Ewes frequently lamb during the winter months, exposing them to cold stress, which can suppress insulin secretion and alter glucose metabolism (Sasaki and Takahashi, 1980) [18]. Conversely, exposure to extreme heat can also disrupt metabolic homeostasis, as observed in cattle (Ulcar and Celeska, 2010) [21]. Serum biochemical parameters, such as glucose, ketone bodies, and mineral content, serve as reliable markers for metabolic disturbances and hepatic insufficiency in these animals (Sykes and Field, 1974; Hajdarevic *et al*., 1989) [19, 6]. Despite the established role of metabolic profiling in assessing ruminant health, limited studies have examined the combined influence of season and reproductive status on serum biochemical markers in sheep.
Follicular fluid plays a crucial role in ovarian physiology, as it serves as a biochemical microenvironment for the developing oocyte. It is composed of serum exudates and locally synthesized metabolites, which reflect the metabolic activity of follicular cells (Gerard *et al*., 2002) [5]. Understanding the composition of follicular fluid is essential for studying folliculogenesis, oocyte maturation, and follicular atresia (Mishra *et al*., 2003) [11]. As oocytes and granulosa cells mature within the follicle, the biochemical composition of follicular fluid undergoes significant changes, influencing the growth and developmental potential of the follicle. Although studies have characterized the biochemical constituents of follicular fluid in various species, including ewes (Wise, 1987) [23], buffaloes (Ahmed *et al*., 1997) [1], goats (Mishra *et al*., 2003) [11], and pigs (Huang *et al*., 2002) [7], comprehensive data on its composition and physiological role in sheep remain scarce. The accumulation of follicular fluid is a critical aspect of follicular growth; however, its biochemical components and their specific roles in ovine follicular development remain poorly understood. Given the close metabolic interplay between serum and follicular fluid, analyzing their biochemical profiles can provide valuable insights into ovarian function and reproductive efficiency in ewes.
The present study aimed to investigate the biochemical composition of serum and follicular fluid in ewes across different age groups (<1 year and >1 year) during two distinct seasons (breeding and non-breeding). Specifically, we examined the concentrations of key metabolites (albumin, bilirubin, calcium, creatinine, glucose, HDL, LDL, cholesterol, magnesium, phosphorus, total protein, triglycerides, and urea) and enzymatic markers (lactate dehydrogenase, SGOT, and SGPT) to identify potential metabolic variations associated with age and seasonal influences. By elucidating these biochemical parameters, this study seeks to contribute to a better understanding of metabolic regulation in relation to ovarian function and reproductive physiology in sheep.

Materials and Methods

Animal Selection and Study Design

A total of 16 ewes were selected from a civil slaughterhouse in Bangalore, India. These animals were divided into two groups based on seasonality: one group (n = 8) was chosen during the breeding season (February-March and July-September), while the other group (n = 8) was selected during the non-breeding season (May-June). The ewes were further categorized into age groups of <1 year and >1 year.
The ewes examined during the breeding season were pregnant and exhibited clinical signs of pregnancy toxemia, including inappetence, ataxia, lethargy, and weight loss. Conversely, ewes studied during the non-breeding season were non-pregnant and clinically healthy.

Ethical Considerations

The study was conducted following ethical guidelines, ensuring that all procedures adhered to institutional and national regulations regarding the humane treatment of animals. Since the samples were obtained from a licensed and civil slaughterhouse, no live animals were harmed for the purpose of this study.

Blood Sample Collection and Processing

Blood samples were collected via jugular venipuncture from each ewe using sterile disposable syringes. Whole blood was collected into plain tubes without anticoagulants and allowed to clot at room temperature for 20–30 minutes. The clot was separated from the container wall using a sterile wooden stick to prevent hemolysis. The samples were then placed in a refrigerator (4 °C) to allow clot retraction. Subsequently, the samples were centrifuged at 2000–3000 rpm for 10 minutes to separate the serum. The collected serum was stored in labeled clean vials at-80 °C until biochemical analysis.

Ovarian Sample Collection and Follicular Fluid Processing

Ovaries were obtained from ewes of different age groups (<1 year, 1-2 years, and >2 years) during both the breeding and non-breeding seasons. The ovaries were transported to the laboratory in warm (32–33 °C) normal saline supplemented with gentamicin (50 µg/mL) within one hour of slaughter.
Follicular fluid was aspirated using a 22-gauge needle attached to a 5 mL plastic syringe while holding the ovaries with forceps. To prevent proteolysis and coagulation, phenylmethylsulfonyl fluoride (PMSF) (20 mg/mL; HiMedia Laboratory, Mumbai, India) and heparin (25 IU/mL; HiMedia Laboratory) were added immediately to the follicular fluid. The collected fluid was centrifuged at 5000 g for 30 minutes at 4 °C to remove cellular debris. The supernatant was then filtered through a 0.2 µm filter (Whatman, Mumbai, India) to ensure purity. The aliquots of follicular fluid were stored at-80 °C until biochemical analysis. Enzymatic assays were performed on freshly collected follicular fluid.

Biochemical Analysis

Serum and follicular fluid samples were analyzed for multiple biochemical parameters, including:

Metabolites: Total protein, albumin, glucose, bilirubin, triglycerides, cholesterol, HDL, LDL, and urea.

Electrolytes and Minerals: Calcium, magnesium, and phosphorus.

Enzymes: Lactate dehydrogenase (LDH), serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT).

All biochemical analyses were performed using commercially available diagnostic kits (Erba, India) following the manufacturer's instructions. Measurements were conducted using a semi-automatic photometer (STAT Fax 3300, Awareness Technology, Inc., USA) based on kinetic and colorimetric methods.

Statistical Analysis

Data were analyzed using SPSS-17 statistical software. One-way analysis of variance (ANOVA) was used to compare the biochemical parameters among different age groups and between seasons. Post-hoc comparisons were conducted using the Bonferroni multiple comparison test (GraphPad Prism, GraphPad Software Inc., San Diego, USA). Differences were considered statistically significant at $p<0.05$.

Results and Discussion

Effect of Age (<1 Year and >1 Year) on the Concentration of Different Biochemical Constituents in Follicular Fluid and Serum

A significant ($p<0.05$) difference was observed in the concentrations of several biochemical constituents between follicular fluid and serum in ewes aged <1 year and >1 year.

Protein and Albumin: The concentration of total protein and albumin was significantly higher ($p<0.05$) in serum compared to follicular fluid in both age groups. This observation aligns with earlier findings, indicating that serum serves as a primary source of protein in follicular fluid (Wise, 1987) [23]. The selective permeability of the blood-follicle barrier regulates the protein influx, and the lower protein concentration in follicular fluid could be attributed to the filtration mechanism that allows only smaller protein molecules to pass through while retaining larger molecules in circulation.

Lipid Profile (HDL, Cholesterol, and Triglycerides): The concentration of HDL and cholesterol was significantly higher ($p<0.05$) in follicular fluid compared to serum, irrespective of age groups. The higher cholesterol levels in follicular fluid can be attributed to the selective permeability of the blood-follicle barrier, which permits the transfer of HDL-bound cholesterol while restricting the movement of larger LDL particles. Additionally, triglyceride concentration was significantly higher ($p<0.05$) in follicular fluid than in serum across both age groups. These findings support the hypothesis that local metabolic processes primarily regulate triglyceride levels in follicular fluid. Despite fluctuations in serum triglyceride levels due to dietary intake and physiological conditions, the ovarian follicle maintains a relatively stable triglyceride concentration.

Urea: Urea concentration was significantly higher ($p<0.05$) in follicular fluid compared to serum in both age groups. This could be due to active transport of urea from blood into the follicular fluid or local synthesis by follicular cells. Similar observations were reported by Collins *et al*. (1997) [24] in mares, where a strong correlation between serum and follicular fluid urea concentrations was noted. The higher urea content in follicular fluid may influence follicular microenvironment and oocyte maturation.

Other Biochemical Constituents: No significant difference ($p>0.05$) was observed in the concentrations of creatinine, bilirubin, glucose, calcium, magnesium, phosphorus, lactate dehydrogenase (LDH), aspartate aminotransferase (SGOT), and alanine aminotransferase (SGPT) between follicular fluid and serum in different aged ewes. This suggests a tightly regulated homeostasis of these metabolites in the follicular environment, independent of age-related variations.

Effect of Breeding Season and Non-Breeding Season on the Concentration of Different Biochemical Constituents in Follicular Fluid and Serum

The present study evaluated the metabolic profile of serum and follicular fluid in ewes across different age groups and breeding seasons. The results provide significant insights into the physiological and biochemical changes occurring due to reproductive status, environmental conditions, and metabolic demands.

Protein and Albumin: The concentrations of total protein and albumin were significantly higher ($p<0.05$) in follicular fluid compared to serum during both breeding and non-breeding seasons. This observation reinforces the role of serum as a major contributor to the follicular fluid protein content (Wise, 1987) [23]. The higher protein concentration in follicular fluid may be crucial for follicular growth and oocyte development, serving as a reservoir of essential amino acids and growth factors.

Creatinine: Serum creatinine levels were significantly higher ($p<0.05$) than follicular fluid levels in both seasons. This indicates that follicular creatinine primarily originates from passive diffusion from the bloodstream. The restricted permeability of the follicular barrier prevents the free movement of larger molecules, resulting in a lower creatinine concentration in follicular fluid compared to serum.

Glucose: The concentration of glucose was significantly higher ($p<0.05$) in serum compared to follicular fluid. The reduced glucose levels in follicular fluid suggest that granulosa cells metabolize glucose efficiently, contributing to local energy production. Since glucose is a crucial energy source for ovarian cells, its metabolism through anaerobic glycolysis results in lactate accumulation. These findings highlight the tightly regulated glucose availability within the follicular microenvironment to support follicular and oocyte maturation.

Calcium and Magnesium Ions: A significantly higher ($p<0.05$) concentration of calcium and magnesium was observed in follicular fluid compared to serum. The inward migration of these ions from circulation and active transport mechanisms may contribute to their elevated levels in follicular fluid. These findings are consistent with previous studies reporting a concentration gradient of cations between serum and follicular fluid, suggesting active

transport mechanisms (Wise, 1987) [23]. Moreover, the lack of direct correlation with serum concentrations suggests that local metabolism may also regulate cation levels in follicular fluid.

Lipid Profile (Triglycerides, HDL, and Cholesterol): The concentration of triglycerides was significantly higher ($p<0.05$) in follicular fluid compared to serum during both breeding and non-breeding seasons. The inability of very low-density lipoproteins (VLDL) to cross the follicular membrane (Grummer and Carroll, 1988) [25] may explain the accumulation of triglycerides in follicular fluid. This suggests that triglycerides serve as an alternative energy source in the follicular environment, as demonstrated by lipid accumulation in oocytes and embryos cultured in triglyceride-rich conditions.

Similarly, the concentration of HDL and cholesterol was significantly higher ($p<0.05$) in follicular fluid compared to serum, irrespective of season. The increased permeability of the follicular wall in mature follicles may allow greater influx of HDL-bound cholesterol, contributing to higher cholesterol levels in follicular fluid. Cholesterol plays a crucial role in steroidogenesis, supporting the biosynthesis of estradiol and other steroid hormones essential for follicular development.

Other Biochemical Constituents: No significant difference ($p>0.05$) was observed in the concentrations of bilirubin, LDL, urea, phosphorus, LDH, SGOT, and SGPT between follicular fluid and serum across different seasons. This indicates that these metabolites are maintained at stable levels, regardless of seasonal variations, highlighting the robustness of follicular homeostasis mechanisms.

Serum Glucose and Energy Metabolism

The observed lower serum glucose levels in pregnant ewes can be attributed to inadequate gluconeogenesis from glucogenic precursors such as propionate, a key byproduct of rumen fermentation. Propionate serves as the primary substrate for hepatic glucose synthesis in ruminants (Wang *et al*., 2012) [22]. However, dietary fluctuations and seasonal food instability, particularly during winter, lead to a deficit of these precursors, compromising glucose production. Consequently, pregnant ewes face an increased risk of energy insufficiency due to heightened fetal demands that are not matched by an increase in endogenous glucose synthesis (Raoofi *et al*., 2013) [15]. The significant increase ($p<0.001$) in β-hydroxybutyrate (BHBA) levels in pregnant ewes compared to their non-pregnant counterparts further supports the presence of a negative energy balance. BHBA, a key ketone body, is an indirect marker of metabolic distress, reflecting the mobilization of adipose tissue reserves in response to energy deficiencies. Seasonal variations also influenced BHBA concentrations, with lower levels recorded in late spring due to improved nutrient availability and enhanced energy supply.

Liver Function and Metabolic Stress

The study also revealed higher aspartate aminotransferase (AST) activity in pregnant ewes ($p<0.05$), suggesting compromised liver function. Increased AST levels indicate hepatic stress, likely due to an excessive metabolic burden and reduced gluconeogenic capacity. The combination of fetal energy demands and limited dietary glycogenic precursors exacerbates hepatic dysfunction during late gestation. This metabolic distress is further aggravated by neuroendocrine changes associated with pregnancy, which disrupt homeostatic regulatory mechanisms. Additionally, environmental stressors such as low ambient temperatures during winter further challenge the energy metabolism of pregnant ewes. Increased metabolic demands, coupled with suboptimal feeding practices and inadequate deworming programs, compromise the animal's ability to cope with energy deficiencies. Serum Protein and Albumin Levels Serum protein levels, particularly albumin, serve as key indicators of hepatic function and nutritional status. A significant decline in serum total protein and albumin levels in pregnant ewes during winter suggests hepatic insufficiency and impaired protein synthesis. Liver dysfunction, coupled with increased metabolic stress, likely contributes to this reduction. Interestingly, pregnant ewes exhibited higher albumin levels during winter compared to non-pregnant ewes. However, this increase may represent pseudo-hyperalbuminemia resulting from dehydration rather than an actual elevation in protein synthesis. Dehydration alters blood protein fractions, leading to artificially elevated albumin levels. In contrast, during late spring, non-pregnant ewes displayed significantly higher serum protein concentrations compared to pregnant ewes, likely due to the consistent availability of dietary protein precursors. Furthermore, proteolysis, triggered by energy shortages, contributed to the lower total protein concentrations in pregnant ewes during winter. Energy deficiencies drive the catabolism of endogenous protein reserves, further exacerbating metabolic imbalances.

The findings of this study highlight the profound impact of pregnancy, seasonality, and metabolic demands on the biochemical profile of ewes. Pregnant ewes experience significant metabolic stress, particularly during winter, due to inadequate gluconeogenesis, heightened ketone body accumulation, hepatic dysfunction, and altered protein metabolism. These challenges underscore the importance of optimizing nutritional management, particularly during late gestation, to prevent metabolic disorders such as pregnancy toxemia. Future research should focus on targeted dietary interventions and metabolic monitoring strategies to enhance reproductive efficiency and overall health in sheep.

Conclusion

This study highlights significant variations in the biochemical composition of follicular fluid and serum in ewes, influenced by age and seasonal factors. The observed differences in protein, lipid, and ion concentrations within the follicular fluid emphasize the selective permeability of the blood-follicle barrier and the role of local metabolic activities in regulating the follicular microenvironment. These findings provide valuable insights into the biochemical dynamics of ovarian follicles, offering potential implications for reproductive physiology and fertility management in ewes. Our results indicate that serum concentrations of total protein, albumin, and creatinine increased with age, while follicular fluid exhibited higher concentrations of HDL, cholesterol, triglycerides, and urea across different age groups. Seasonal variations also played a crucial role, with follicular fluid showing elevated levels of total protein, albumin, HDL, cholesterol, triglycerides, calcium, and magnesium compared to serum. Conversely, serum exhibited higher concentrations of creatinine and

glucose relative to follicular fluid across different breeding seasons. These biochemical variations suggest that the metabolic environment surrounding developing oocytes and granulosa cells is closely linked to systemic metabolic changes. The influence of these metabolic alterations on oocyte quality across different seasons, follicular phases, and age groups in sheep warrants further investigation. Future studies should explore how these biochemical changes impact follicular development, oocyte competence, and overall reproductive efficiency in ewes, which could provide essential insights for improving breeding strategies and fertility outcomes.

Table 1: Follicular Fluid and serum in relation to age:

	Follicular fluid		Serum	
Metabolite	**< 1 yr,**	**> 1 yr**	**< 1 yr,**	**> 1 yr**
Total protein (g/dl)	4.63±0.43	4.82±0.78	5.55±1.73*	5.79±0.23*
Albumin (g/dl)	2. 15±0.63	2.34±0.34	3.21±1.39**	3.78±1.34**
Creatinine (mg/dl)	0.59±0.13	0.73±0.12	0.64±0.10*	0.98±0. 14*
Bilirubin (mg/dl)	0.23±0.03	0.29±0.08	0.25±0. 12*	0.31±0.13
Glucose (mmol/l)	1.05±0.19	1.37±0.21	1.31±0. 78	1.74±0.98
HDL (mg/dl)	48.32±8.54	49.76±9.32	45.57±9.42*	46.65±8.45**
LDL (mg/dl)	10.99±7.54	12.32±2.65	10.45±1.21	12.66±4.34
Cholesterol (mg/dl)	37.76±8.21	38.96±7.86	76.23±10.43**	78.76±9.43**
Triglycerides (mg/dl)	32.56±7.98	35.85±7.43	28.32±7.73*	29.73±5.56*
Urea (mmol/l)	5.00±1.43	5.12±1.32	4.23±1.37	4.65±1.87
Calcium (mmol/l)	2.39±0.54	2.90±0.54	2.43±1.13	2.89±2.56
Magnesium (mmol/l)	1.12±0.76	1.98±0.65	1.96±1.34	1.16±0.45
Phosphorous (mmol/l)	1.21±0.45	1.65±0.87	1.34±0.53	1.98±0.97
Lactate dehydrogenase (U/I)	240.45±23.56	245.63±20.53	241.12±19.32	245.13±24.23
SGOT (U/I)	78.34±12.78	79.58±11.23	79.10±9.92	80.36±12.78
SGPT (U/I)	26.23±13.32	26.67±8.31	26.87±6.32	26.95±7.70

Data expressed as Mean ± SD, In each row value with different letters are significant in relation follicular fluid and serum of different age groups

Table 2: Follicular Fluid and serum in relation to breeding season and non breeding season

	Follicular fluid		Serum	
Metabolite	**Breeding season**	**Non breeding season**	**Breeding season**	**Non breeding season**
Total protein (g/dl)	5.83±0.20	5.42±0.64	4.25±1.23*	4.76±0.54*
Albumin (g/dl)	2. 35±0.57	2.14±0.49	3.43±1.76*	3.87±1.05*
Creatinine (mg/dl)	0.40±0.11	0.53±0.19	0.94±0.21*	0.98±0.32*
Bilirubin (mg/dl)	0.27±0.06	0.30±0.06	0.34±0. 10	0.37±0.16
Glucose (mmol/l)	1.25±0.17	0.98±0.32	2.32±0. 67**	1.87±0.78**
HDL (mg/dl)	47.12±7.94	45.36±8.32	46.37±8.76*	43.43±8.76*
LDL (mg/dl)	11.69±7.65	13.84±2.72	11.43±1.76	13.19±5.12
Cholesterol (mg/dl)	43.34±7.43	37.54±8.54	40.57±11.32*	35.45±10.67*
Triglycerides (mg/dl)	27.75±8.33	26.98±6.98	24.67±8.12*	22.77±6.12**
Urea (mmol/l)	5.87±0.98	5.54±1.09	4.32±1.54*	4.18±1.54*
Calcium (mmol/l)	3.79±0.34	3.90±0.67	2.98±0.45*	2.29±3.55*
Magnesium (mmol/l)	2.26±0.85	2.39±0.77	1.36±0.67	1.45±0.76
Phosphorous (mmol/l)	1.91±0.52	1.74±0.97	1.88±0.22	1.79±0.56
Lactate dehydrogenase (U/I)	456.32±27.43	187.65±19.45	486.78±18.45*	199.45±21.55
SGOT (U/I)	84.75±13.33	85.43±12.34	84.66±10.66*	84.22±14.65
SGPT (U/I)	29.94±12.85	30.65±7.67	30.14±7.65*	30.76±8.32

Data expressed as Mean ± SD, In each row value with different letters are significant in relation to follicular fluid and serum of breeding season and non breeding season

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