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Evaluation of leukocyte count and biochemical constitutes including oxidant-antioxidant indices in cyclic and acyclic ewes with or without uterine infection

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Abstract
The present study was conducted at Faculty of Veterinary Sciences and Animal Husbandry, Shuhama to determines the alterations in leukocyte and biochemical constituents including oxidant-antioxidant indices in the blood serum of cyclic and acyclic ewes with or without uterine infection. Both infectious and normal ewes were further sub divided into Follicular infectious (FI), Luteal infectious (LI), Follicular normal (FN), Luteal normal (LN) and Acyclic normal (AC). The mean pH of the uterine secretions obtained from ewes suffering from uterine infection was significantly higher (P<0.001) than normal ewes. Neutrophils and TLC in the infectious group was significantly higher than in the normal group of ewes. The NO concentration in follicular fluid was significantly higher (P<0.001) in FI (56.91±11.94) and LI (51.25±8.75) group than FN (11.64±0.96), LN (14.79±2.40) and AC (18.67±1.13) group of ewes. Follicular ascorbate was significantly lower (P<0.05) in FI (30.85±6.28) than FN (73.20±12.16) and AC (63.05±15.09) ewes. Follicular cholesterol concentration was significantly higher (P<0.05) in LI (133.03±1.25) than FN (129.16±0.44), LN (129.96±0.24) and AC (129.42±0.51) ewes. Total protein concentration (g/dl) was significantly higher (P<0.05) in LN than both luteal group of ewes (LN and LI). The mean concentration of serum MDA is shown in Table 8 and Fig 9. The circulatory MDA level (nmol/L) was significantly higher (P<0.05) in FI (1049.32±25.64) and LI group (1030.25±26.69) than AC (952.95±15.55) group of ewes**.** The Serum Mn level was significantly higher (P<0.05) in LI (7.31±0.49) and LN (6.26±1.13) than FI (3.01±0.98) group of ewes. In general, the zinc concentration was observed to be higher in cyclic normal ewes as compared to cyclic infectious ewes.

Keywords: Cyclic and acyclic ewes, uterine infection, leukocyte count, ascorbic acid concentration, serum cholesterol concentration, malondialdehyde level

1. Introduction
Sheep (*Ovis aries*) are quadrupedal ruminant species mostly raised for mutton in our condition but the seasonal pattern of this species under the temperate climate conditions of Jammu and Kashmir limits its production as only one lamb crop is obtained per year [1]. The mutton production is further limited if some ewes become infertile and turn out to be dry at the end of the breeding season. The "dry ewe" percentage in an organised farm of Kashmir has been found to be 23.62% [1]. Uterine infection, an important cause of infertility, has been reported to be 5 to 10% in sheep [2] and 9.3% in goats [3]. Despite some published reports, the "true" incidence of uterine infection is not known for any livestock species including sheep because the detection and diagnosis are often inaccurate and reporting is not mandatory [4]. The term 'uterine infection' implies adherence of pathogenic organisms to the mucosa, colonization or penetration of the epithelium and/or release of bacterial toxins that lead to establishment of uterine diseases [5]. Pathogenic bacteria pass through the cervix and contaminate the uterus resulting in endometritis [5]. Many methods are used for the diagnosis of endometritis including inspection of vaginal discharge, transrectal palpation, transrectal ultrasonography, uterine bacterial culture, uterine biopsy and endometrial cytology [6]. Infection and inflammatory conditions greatly affect fertility in animals. In cattle and buffalo the studies on the diagnosis and therapeutic management of infertility have been conducted systematically and [7]. Blood metabolic profile (BMP) is a set of diagnostic procedures that are based on determining the various indicators in the blood of animals [8]. The most common indicators in the blood of

animals used in the preparation of the BMP are biochemical and haematological parameters. BMP is used in assessing nutritional status and animal health [9]. Blood indices may vary depending on factors such as sex, age, weather, stress, season and physical exercise [10]. Cholesterol plays an important role in reproduction as a precursor for the biosynthesis of steroids [11]. Cholesterol in the ovary can be derived from two sources: cellular *de novo* synthesis from acetate or uptake of plasma lipoprotein cholesterol. Avascularised granulosa cells are restricted to cholesterol uptake from HDL. Although the major precursor for estrogen production by granulosa cells is believed to be androgen derived from thecal cells [12], increased granulosa cell progesterone production occurring after the LH surge and prior to vascularization [13] may be dependent on the supply of sterol precursor from follicular fluid lipoproteins. Vascularized steroidogenic tissues such as theca interna and corpus luteum may utilize cholesterol from either of the two major cholesterol carrying lipoproteins (i.e, LDL or HDL). It is known that blood cholesterol concentrations and steroid hormones synthesis are positively related to energy intake and health of animals as lower cholesterol and glucose concentrations after calving have been associated with an increased number of days from calving to conception [14].

Nitric oxide (NO) is an inorganic signaling molecule that diffuses freely through biological membranes and found to be an important mediator involved in regulating the reproductive and immune functions in mammals. Dairy cows with uterine infection had higher NO concentrations in plasma and uterine secretions [15]. The involvement of NO in the modulation of ovarian function is documented by several studies aimed at demonstrating its production within the ovary and at clarifying its role in the regulation of steroidogenesis, follicle development, oocyte maturation, ovulation, luteal function, and luteal regression [16]. Recently an increased level of NO and progesterone in follicular fluid of buffalo suffering from endometritis has been reported [17]. However, luteal NO concentration has been found to be decreased in buffalo cows suffering from endometritis [18]. Ascorbic acid (Vitamin C), a multifunctional antioxidant has been found in follicular fluid, ovarian tissue and corpus luteum (CL) of buffalo [19]. Its functions at ovarian level are multifaceted, including remodeling of gonadal tissue due to its role in collagen synthesis [20], biosynthesis of steroid hormones [21] and as a part of the ovarian antioxidant system [22]. The vitamin C content in the CL is at its maximum when the CL is fully mature, remains high during pregnancy, and decreases as the CL regresses [23].

Cell membranes are rich sources of polyunsaturated fatty acids (PUFAs). The oxidative destruction of polyunsaturated fatty acids (PUFAs) is known as lipid peroxidation which causes tissue injury through the production of reactive oxygen species (ROS). Lipid peroxidation is known to have a role in aging, cancer and many infectious diseases. Malondialdehyde (MDA), a by-product of lipid peroxidation, is used as an index of the rate of tissue reaction chain. MDA is also used as an indicator of oxidative stress in cells and tissues [24, 25]. The mechanism of damage involves lipid peroxidation, which destroys cell membranes with the release of intracellular components, such as lysosomal enzymes, leading to further tissue damage [26, 27] studied serum nitric oxide and malondialdehyde levels in cattle infected with *Brucella abortus* and concluded that increased MDA may be a result of excessive production of radical secondary to brucellosis itself acting upon membrane lipids leading to tissue damage. MDA levels in cattle infected with brucellosis were significantly higher than the control group ($p < 0.01$). [28] reported that MDA level is greatly increased in cows naturally infected with *N. caninum*. The higher blood MDA concentrations in cows affected with endometritis as compared to healthy animals is apparently due to a marked increase in ROS production during development of the inflammatory process [29]. Significantly higher MDA level was detected in cows suffering from postpartum uterine diseases like clinical metritis and endometritis [30]. Greater MDA level in cyclic cows with subclinical endometritis compared to non-endometritis cows has also been reported [31]. Minerals are the essential nutrients bearing a significant role in the animal reproduction, because their excess or deficiency produces detrimental effect on the performance of livestock [32]. They influence the physiology of reproduction through their actions as metalloproteins. Deficiency of these elements leads to impaired reproductive performance in mammals [33]. Dietary deficiency of minerals results in poor reproductive performance leading to infertility, late puberty, abortion and repeat breeding in animals. Trace elements may function as cofactors of enzymes, or stabilizers of secondary molecular structure. There has been special interest in effects of dietary trace element deficiencies on physiological functions and particularly on reproduction. Severe dietary deficiencies of copper, selenium and zinc are commonly seen in ruminants [34]. The supplementation of zinc oxide to the basal diet of Baladi ewes significantly improved the reproductive performance [35]. Manganese functions as a cofactor for a large variety of enzymes including enzymes involved in reproductive functions. A deficiency in manganese can inhibit the synthesis of cholesterol, which may limit the production of hormones essential for reproduction [36]. Nitric oxide (NO) is an inorganic signalling molecule that diffuses freely through biological membranes. It has been found to be an important mediator involved in regulating the reproductive and immune functions in mammals. Dairy cows with uterine infection had higher NO concentrations in plasma and uterine secretions [15]. The involvement of NO in the modulation of ovarian function is documented by several studies aimed at demonstrating its production within the ovary and at clarifying its role in the regulation of steroidogenesis, follicle development, oocyte maturation, ovulation, luteal function, and luteal regression [37]. Recently an increased level of NO and progesterone in follicular fluid of buffalo suffering from endometritis has been reported [17]. However, luteal NO concentration has been found to be decreased in buffalo cows suffering from endometritis [18]. Ascorbic acid (Vitamin C), a multifunctional antioxidant has been found in follicular fluid, ovarian tissue and corpus luteum (CL) of buffalo [19]. Its functions at ovarian level are multifaceted, including remodelling of gonadal tissue due to its role in collagen synthesis [20], biosynthesis of steroid hormones [21] and as a part of the ovarian antioxidant system [22]. Therefore it is important to know the incidence of infertility due to infectious or inflammatory conditions of the uterus along with their effect on the biochemical constituents to understand the possible measures for its amelioration in animals. Perusal of available literature revealed that information on biochemical and hormonal level in ewes suffering from uterine infections in relation to cyclicity are not available. Keeping in view the above facts, this study was designed with to study the leukocyte count and biochemical constituents including the

oxidant-antioxidant indices in cyclic and acyclic ewes with or without uterine infections.

2. Materials and Methods

The study was conducted during May to October 2013, in female sheep that were brought for slaughter in the local abattoirs. The age, breed, body condition of the animal was recorded and clinico-gynaecological examination of the animal was performed to select the animals without any apparent systemic diseases. At the time of slaughter, blood was collected from ninety two ewes in two parts. Blood sample collected in heparinised (20 i.u/ml) tubes was used for estimation of leukocyte profile and separation of plasma. In another centrifuge tube whole blood was allowed to clot in slanting position and subsequently centrifuged for separation of serum. The serum samples were stored at -20ºC till further analysis. Female genitalia were procured from all ewes after slaughter at the local abattoir and transported in Phosphate Buffer Saline (PBS) in Ice Chest for further processing and diagnosis. Out of the total ewes, 66 were subjected for further investigation in respect circulatory leukocyte count and biochemical constituents indices. Upon reaching the laboratory, the genital tracts were examined for any gross abnormality and then opened longitudinally from the cervical end to the oviductal end of both horns with the help of scissors. The endometrial washing was collected and subjected to the following tests for diagnosis of uterine infection. The identification of tracts for uterine infection was done with the help of white side test, pH of uterine washings followed by endometrial cytology. The identified tracts were divided into two groups- positive (n=24) or negative (n=68) for uterine infection.

2.1 Total leukocytic count (TLC) as per routine procedure.

2.2 Differential leukocyte count (DLC) as per routine procedure.

2.3 Estimation of biochemical profile

2.3.1 Total Protein as per routine procedure.

2.3.2 Ascorbic Acid: The ascorbic acid concentration in serum, follicular fluid and CL was determined as per the method described of Zannoni *et al*. (1974) [38].

2.3.3 Nitric Oxide: Nitric oxide concentration in serum, follicular fluid and CL was determined by the method described by Sastry *et al*. (2002) [39].

2.3.4 Malondialdehyde (MDA): Malondialdehyde (MDA) levels, an index of lipid peroxidation were measured by the double heating method of Draper and Hadley (1990) [44].

2.3.5 Zinc and Manganese: The estimation of Zinc and Manganese was done by Atomic Absorption Spectrophotometer.

2.4 Statistical Analysis

The data for leukocyte count and biochemical constituents were analyzed by one way ANOVA for comparison between groups using SPSS (14) version for windows. The data pertaining to a particular parameter for comparison between two groups in respect of corpus luteum were compared using independent sample T test. If a main effect was found significant, post hoc analysis was performed with LSD. The values were considered as significant at $P<0.05$. The data are presented as Mean ± S.E.M.

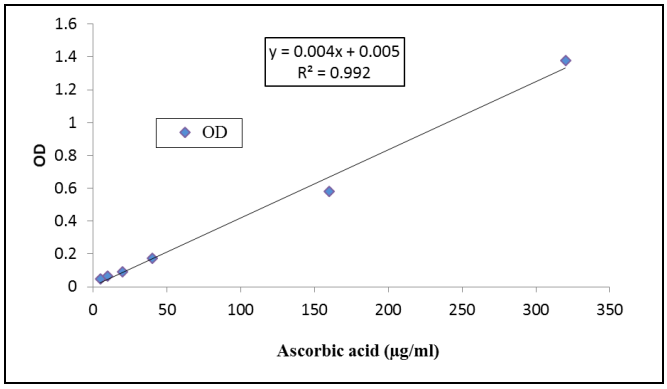


Fig 1: Standard curve for estimation of Ascorbic Acid

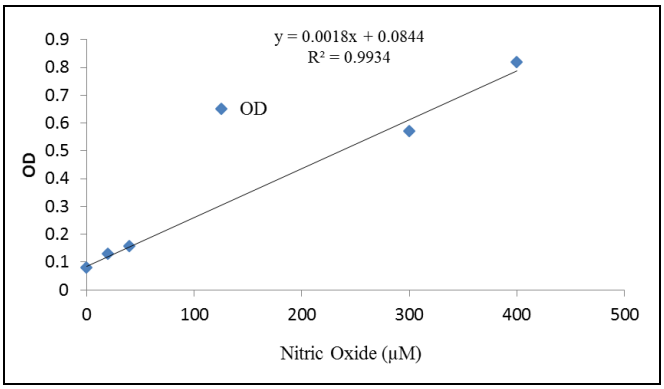


Fig 2: Standard curve for estimation of Nitric Oxide

3. Results

A total of 92 genitalia were examined, out of which 24 (26.09%) were detected positive while 68 (73.91%) were found negative for uterine infection based on the white side test, pH of uterine washings and endometrial cytology. On the basis of the functional structures present on the ovaries, both infectious and normal ewes were further sub divided into Follicular infectious (FI), Luteal infectious (LI), Follicular normal (FN), Luteal normal(LN) and Acyclic normal (AC). The percentage of ewes in FI, LI, FN, LN and AC groups was 6.52%, 19.56%, 23.91%, 17.39% and 32.60%, respectively (Table 1). The total leukocyte count (TLC) in blood was significantly ($P<0.05$) higher in LI (10.03±0.43) than FN (4.96±0.77), LN (5.47±0.73) and AC (6.04±0.53) group of ewes (Table 2 and Fig 3). The mean differential leukocyte count (DLC) is presented in Table 3 and Fig 4. The mean neutrophils percentage was significantly higher ($P<0.001$) for luteal LI (48.50±0.84) and FI (45.33±1.58) than FN (25.00±1.71), LN (20.66±1.14) and AC (26.50±2.43) group of ewes. The mean lymphocyte concentration was significantly higher ($P<0.001$) for LN (71.00±1.31) and FN (67.16±1.99) and AC (66.00±2.47) than LI (35.83±1.07), FI (40.00±1.29) group of ewes. The mean neutrophils lymphocyte ratio was significantly higher in LI (1.36±0.05) and FI (1.14±0.07) as compared to FN (0.37±0.03), LN (0.29±0.02) and AC (0.41±0.05) group of ewes.

The mean concentration of nitric oxide (NO) in serum, follicular fluid and corpus luteum of different group of ewes is presented in Table 4 and Fig 5. The NO concentration (µM) in serum was significantly higher ($P<0.05$) in AC (24.80±1.48) as compared to LN (9.58±1.78), FN (9.75±3.05), FI (14.16±2.55) and LI (16.16±2.78) group. The NO concentration in follicular fluid was significantly higher ($P<0.001$) in FI (56.91±11.94) and LI (51.25±8.75) group

than FN (11.64±0.96), LN (14.79±2.40) and AC (18.67±1.13) group of ewes. Nitric oxide concentration in corpus luteum did not differ significantly between LI (28.40±2.76) and LN (29.90 ± 2.92) ewes. The follicular NO was significantly higher ($P< 0.001$) in LI and FI and lower ($P< 0.05$) in AC group of ewes than the concentration in serum. Whereas in LN ewes, luteal NO concentration was significantly higher ($P<0.001$) than follicular fluid and serum (Table 4). The mean concentrations of ascorbic acid recorded in infectious and normal group of ewes are presented in Table 5 and Fig 6. The mean concentration of serum ascorbate (µg/ml) was significantly lower ($P<0.05$) in LI (11.90±1.72) than in FN (46.21±9.26) and LN (34.02±7.51) ewes. Follicular ascorbate was significantly lower ($P<0.05$) in FI (30.85±6.28) than FN (73.20±12.16) and AC (63.05±15.09) ewes. The difference in luteal ascorbate was not significant between LI (13.62± 2.26) and LN (15.65 ± 1.03) group of ewes. It is interesting to note that follicular ascorbic acid concentration was higher in all groups of ewes than in serum and CL (Table 5). The mean cholesterol concentration is shown in Table 6 and Fig 7. Serum cholesterol concentration (mg/ml) was significantly higher ($P<0.05$) in LN (163.71±0.83) as compared to LI (157.06±1.93), FN (151.93±3.06) and AC (156.14±1.51) ewes. Follicular cholesterol concentration was significantly higher ($P<0.05$) in LI (133.03±1.25) than FN (129.16±0.44), LN (129.96±0.24) and AC (129.42±0.51) ewes. The mean concentration of follicular cholesterol was significantly higher ($P<0.05$) in infectious animals as compared to normal animals. Irrespective of cyclic status and infectious condition of uterus cholesterol concentration was significantly higher ($P<0.001$) in serum (151.93±3.06 to 163.71±0.83) than the corresponding values in follicular fluid (129.16±0.44 to 133.03±1.25) of all groups of ewes (Table 6). The mean concentration of serum total protein is shown in Table 7 and Fig 8. Total protein level (g/dl) in serum was significantly higher ($P<0.05$) in FN (5.72±0.14) than LN (5.44±0.04) and LI (5.37±0.06) group of ewes. The FI group of ewes also showed significantly higher serum total protein than LI. The mean concentration of serum MDA is shown in Table 8 and Fig 9. The circulatory MDA level (nmol/L) was significantly higher ($P<0.05$) in FI (1049.32±25.64) and LI group (1030.25±26.69) than AC (952.95±15.55) group of ewes**.** The mean concentration of Zinc (ppm) in serum is presented in Table 9 and Fig 10. The Serum Zn level was significantly higher ($P<0.001$) in LN (3.36±0.65) than FI (1.53±0.42), AC (1.61±0.20) and LI (1.70±0.31) group of ewes. In general, the zinc concentration was observed to be higher in cyclic normal ewes as compared to cyclic infectious ewes. The mean concentration of Manganese (ppm) in serum is presented in Table 10 and Fig 11. The Serum Mn level was significantly higher ($P<0.05$) in LI (7.31±0.49) and LN (6.26±1.13) than FI (3.01±0.98) group of ewes. Irrespective of infectious condition of uterus, luteal group of ewes showed higher Mn level than the follicular group.

Table 1: Distribution of experimental ewes

Total no. of samples N=92				
Infectious (n=24)		**Normal (n=68)**		
26.08%		73.91%		
FI (n=6)	LI (n=18)	FN (n= 22)	LN (n= 16)	AC (n=30)
6.52%	19.56%	23.91%	17.39%	32.60%

AC=Acyclic; FN= Follicular normal; FI= Follicular infectious; LN= Luteal normal; LI= Luteal infectious

Table 2: Total Leukocyte Count (TLC) in ewes with or without uterine infections (mean ± SEM)

Groups	TLC(x 10^3 / µl)
AC	$6.04±0.53^{a}$
FI	$7.58±1.72^{ab}$
FN	$4.96±0.77^{a}$
LI	$10.03±0.43^{b}$
LN	$5.47±0.73^{a}$

Means bearing different superscripts (a, b) within a column differ significantly ($P<0.05$)

AC=Acyclic; FN= Follicular normal; FI= Follicular infectious; LN= Luteal normal; LI= Luteal infectious.

Table 3: Differential Leukocyte Count (DLC) in ewes with or without uterine infections (mean ± SEM)

Groups	DLC		
	Neutrophils (N)	**Lymphocytes (L)**	**N : L RATIO**
AC	$26.50±2.43^{b}$	$66.00±2.47^{b}$	$0.41±0.05^{a}$
FI	$45.33±1.58^{c}$	$40.00±1.29^{a}$	$1.14±0.07^{b}$
FN	$25.00±1.71^{ab}$	$67.16±1.99^{b}$	$0.37±0.03^{a}$
LI	$48.50±0.84^{c}$	$35.83±1.07^{a}$	$1.36±0.05^{c}$
LN	$20.66±1.14^{a}$	$71.00±1.31^{b}$	$0.29±0.02^{a}$

Means bearing different superscripts (a, b, c) within a column differ significantly ($P<0.001$)

AC=Acyclic; FN= Follicular normal; FI= Follicular infectious; LN= Luteal normal; LI= Luteal infectious.

Table 4: Nitric oxide concentration (mean ± SEM) in ewes with or without uterine infections

Groups	Nitric Oxide(µM)		
	Serum*	**Follicular fluid****	**Corpus luteum**
AC*	$24.80±1.48^{bB}$	$18.67±1.13^{aA}$	-
FI*	$14.16±2.55^{aA}$	$56.91±11.94^{bB}$	-
FN*	$9.75±3.05^{a}$	$11.64±0.96^{a}$	-
LI*	$16.16±2.78^{aA}$	$51.25±8.75^{bB}$	$28.40±2.76^{A}$
LN**	$9.58±1.78^{aA}$	$14.79±2.40^{aA}$	$29.90±2.92^{B}$

Means bearing different superscripts (a, b, c) within a column differ significantly; $^{*}(P<0.05)$, $^{**}(P<0.001)$; (A,B) within rows differ significantly $^{*}(P<0.05)$, $^{**}(P<0.001)$

AC=Acyclic; FN= Follicular normal; FI= Follicular infectious; LN= Luteal normal; LI= Luteal infectious

Table 5: Ascorbic acid concentration (mean ± SEM) in ewes with or without uterine infections

Groups	Ascorbic acid(µg/ml)		
	Serum	**Follicular fluid**	**Corpus luteum**
AC	$23.00±5.46^{abA}$	$63.05±15.09^{bB}$	-
FI	$24.73±3.69^{ab}$	$30.85±6.28^{a}$	-
FN	$46.21±9.26^{c}$	$73.20±12.16^{c}$	-
LI	$11.90±1.72^{aA}$	$32.80±5.63^{abB}$	$13.62±2.26^{A}$
LN	$34.02±7.51^{bcA}$	$56.77±7.72^{abcB}$	$15.65±1.03^{A}$

Means bearing different superscripts (a, b, c) within a column differ significantly ($P<0.05$); (A, B) within rows differ significantly ($P<0.05$).

AC=Acyclic; FN= Follicular normal; FI= Follicular infectious; LN= Luteal normal; LI= Luteal infectious

Table 6: Cholesterol concentration (mean ± SEM) in ewes with or without uterine infections

Groups	Cholesterol (mg/ml)	
	Serum	Follicular fluid
AC	156.14±1.51^{abB}	129.42±0.51^{Aa}
FI	159.43±2.37^{bcB}	132.28±1.23^{bcA}
FN	151.93±3.06^{aB}	129.16±0.44^{aA}
LI	157.06±1.93^{abB}	133.03±1.25^{cA}
LN	163.71±0.83^{cB}	129.96±0.24^{abA}

Means bearing different superscripts (a, b, c) within a column differ significantly (P<0.05); (A, B) within rows differ significantly (P<0.001).
AC=Acyclic; FN= Follicular normal; FI= Follicular infectious; LN= Luteal normal; LI= Luteal infectious

Table 7: Total Protein concentration (mean ± SEM) in ewes with or without uterine infections

Groups	Total Protein(g/dl)
	Serum
AC	5.6217±0.04^{bc}
FI	5.6533±0.05^{bc}
FN	5.7233±0.14^{c}
LI	5.3767±0.06^{a}
LN	5.4433±0.04^{ab}

Means bearing different superscripts (a, b, c) within a column differ significantly (P<0.05)
AC=Acyclic; FN= Follicular normal; FI= Follicular infectious; LN= Luteal normal; LI= Luteal infectious

Table 8: Malondialdehyde concentration (mean ± SEM) in ewes with or without uterine infections

Groups	MDA(nmol/l)
	Plasma
AC	952.95±15.55^{a}
FI	1049.32±25.64^{b}
FN	986.11±22.62^{ab}
LI	1030.25±26.69^{b}
LN	1009.67±19.06^{ab}

Means bearing different superscripts (a, b) within a column differ significantly (P<0.05)
AC=Acyclic; FN= Follicular normal; FI= Follicular infectious; LN= Luteal normal; LI= Luteal infectious

Table 9: Concentration of trace minerals (mean ± SEM) in the serum of ewes with or without uterine infections

Groups	Serum	
	Zn (ppm)	Mn (ppm)
AC	1.61±0.20^{a}	5.75±0.57^{bc}
FI	1.53±0.42^{a}	3.01±0.98^{a}
FN	2.35±0.29^{ab}	4.11±0.54^{ab}
LI	1.70±0.31^{a}	7.31±0.49^{c}
LN	3.36±0.65^{b}	6.26±1.13^{bc}

Means bearing different superscripts (a, b) within a column differ significantly (P<0.05) AC=Acyclic; FN= Follicular normal; FI= Follicular infectious; LN= Luteal normal; LI= Luteal infectious

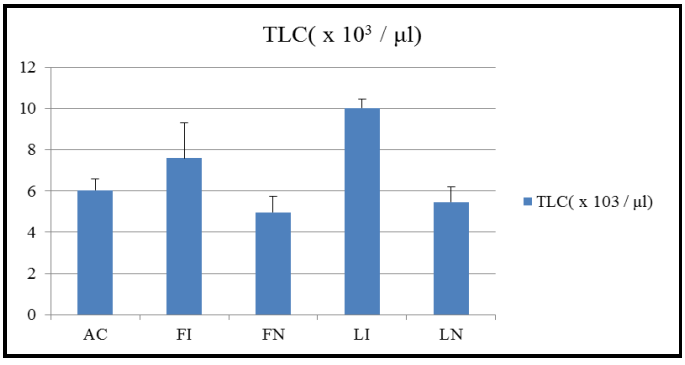


Fig 3: Total Leukocyte Count (TLC) in ewes with or without uterine infections

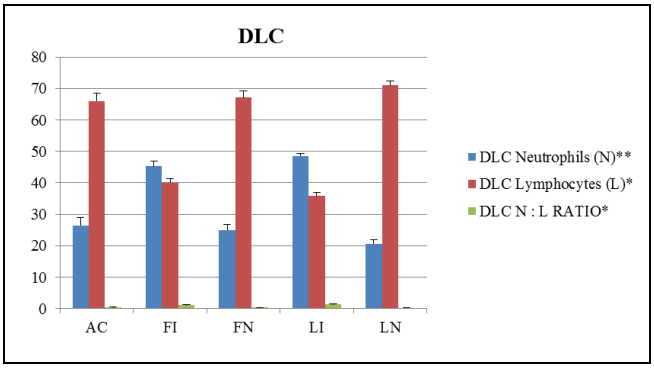


Fig 4: Differential Leukocyte Count (DLC) in ewes with or without uterine infections

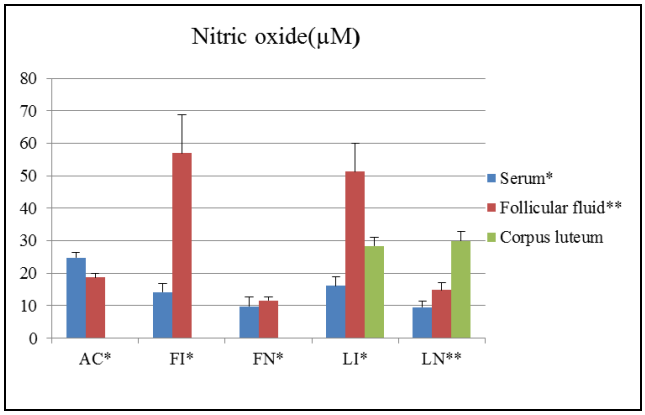


Fig 5: Nitric oxide concentration in ewes with or without uterine infections

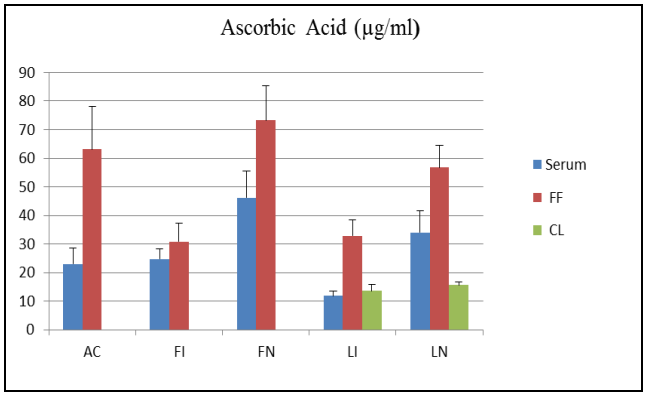


Fig 6: Ascorbic acid concentration (mean ± SEM) in ewes suffering from uterine infections

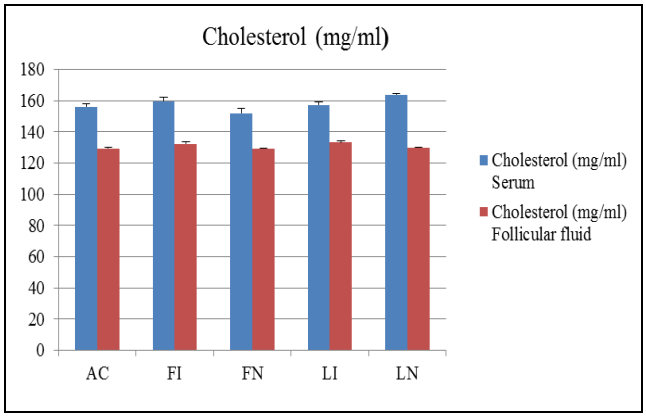


Fig 7: Cholesterol concentration in ewes with or without uterine infections

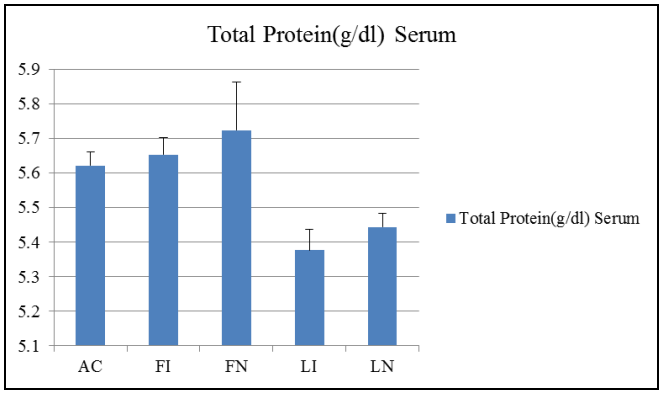


Fig 8: Total Protein concentration (mean ± SEM) in ewes with or without uterine infections

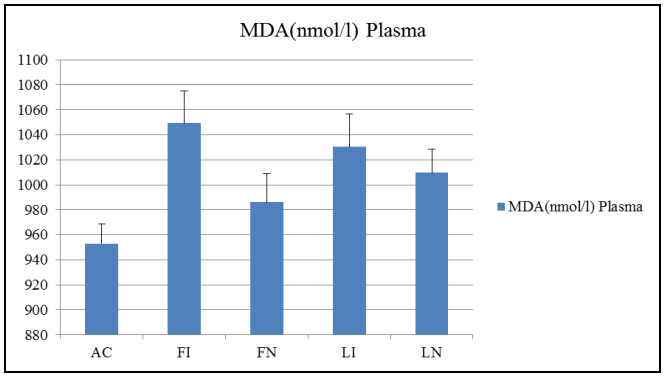


Fig 9: Malondialdehyde concentration in ewes with or without uterine infections

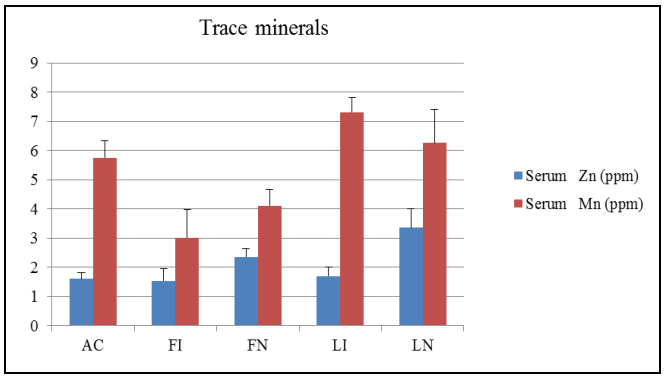


Fig 10: Concentration of trace minerals (mean ± SEM) in the serum of ewes with or without uterine infection

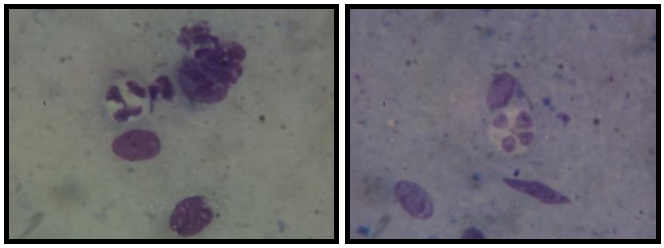
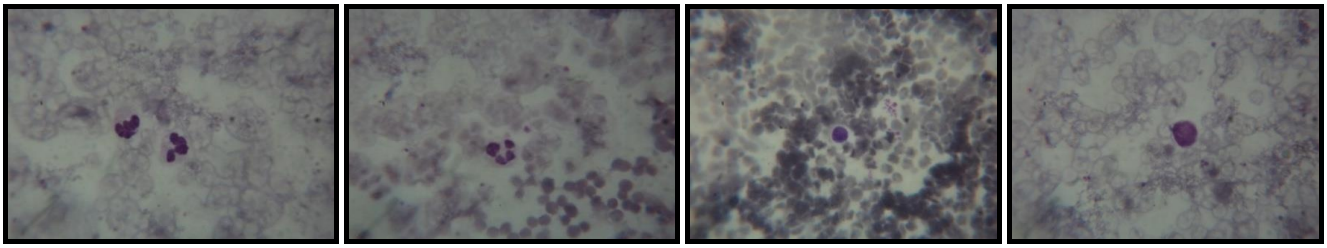


Plate 1: Endometrial cytology showing neutrophils



Neutrophils Lymphocyte

Plate 2: Differential Leukocyte Count (DLC)

3. Discussion

The uterine infection in ewes (26.08%) recorded in this study was in accordance with the previous reports in cattle [40] and Buffalo [41]. The highest incidence of uterine infection in present study may take support from the findings of [42] who reported higher incidence in clinical cases of uterine infection on the basis of detailed etiological background. The higher percentage of infectious uterine condition recorded in this study warrants special investigation in ewes to ameliorating the infertility condition. This study showed higher incidence of acyclic ewes (32.60%) which is in line with the incidence of dry ewes reported by [30] in Corriedale ewes at an organized farm of Kashmir. However, the incidence of dry ewe percentage in crossbred Merino sheep in farmers flock of migratory sheep [43] and anestrus in Muzaffarnagari flock of ewes [44] was lower than the present study. The difference in incidence of uterine infection and acyclic ewes in the present study compared to earlier reports might also be attributed to the variations in species, age, breeds of animals, diagnostic procedures applied for the study and also agro-climatic conditions of the area of investigation. The significantly higher TLC recorded in LI than FN, LN and AC group in the present study may take support from the previous reports in dairy cattle suffering from post partum uterine infections [45].

The higher TLC recorded in the ewes suffering from uterine infections may be attributed to the increased circulating neutrophils in these ewes recorded in this study. The higher TLC in the ewes suffering from uterine infections is in accordance with the findings of [46] in cows with post partum endometritis. The higher neutrophil percentage and TLC might also be due to the increased cortisol level in the ewes as the MDA level in these ewes was recorded higher than the normal ewes. The simultaneous increase in MDA and cortisol level in the cows suffering postpartum metritis and endometritis compared to normal cows has already been reported [30]. Corticosteroids induce neutrophilia by an increased output of neutrophils from the bone marrow, by neutrophils demargination from the blood vessel wall, or by a combination of the two [47]. The significantly higher neutrophils percentage and neutrophil lymphocyte ratio observed in LI and FI than FN, LN and AC group was also reported by [48] and Islam [30] in postpartum cows suffering from uterine infections.

The significantly higher follicular fluid NO in FI and LI than FN, LN and AC group observed in the present study is in agreement with the finding of [17] in the follicular fluid of infected buffaloes. However, the difference in the level of NO between the cyclic and acylic normal ewes was not significant in this study as reported earlier by [49] in follicular fluid of sheep. In LN group, the luteal NO concentration was significantly higher than in follicular fluid and serum which, may be due to rapid vascularization of initially avascular granular lutein cell tissue for the formation of mature functional CL [56]. The higher NO level in the follicular fluid of the cyclic group of ewes than the serum of these ewes might be attributed to its role within the ovary for various reproductive events - as an important intra-ovarian factor, NO regulates the process of follicular development by its multifaceted role in angiogenesis, vasodilation and regulation of follicular basement membrane permeability, steroidogenesis, ovulation, atresia and luteolysis [50, 51]. There is now compelling evidence that NO is one of the many intra-ovarian mediators which affects on the ovulatory process and regulation of corpus luteum function [52]. The higher No concentration in the follicular fluid and serum of acyclic ewes than the normal cyclic (FN and LN) observed in this study is in concurrence with the previous report in the follicular fluid of buffalo [53]. The higher level of No in the acyclic normal ewes than the normal cyclic ewes indicates that follicular development continues during acyclicity in ewes as it was earlier found in acyclic buffaloes [53]. The presence of an intra follicular and luteal NO generating system at all stages of cyclic infected as well as healthy ewes imply that NO plays critical physiological role in the regulation of ovarian function during oestrous cycle and also in pathological condition like uterine infection is in line with the previous in buffaloes [53, 17, 18]. The lower ascorbic acid level in both serum and follicular fluid of cyclic infectious group of ewes (LI and FI) than cyclic and acyclic normal group of ewes recorded in the present study was in accordance with the previous reports [17]. Ascorbic acid may play an important role in scavenging ROS and serves as an important part of the ovarian antioxidant system [54]. The decreased ascorbic acid concentration in the follicular fluid and serum of ewes suffering from uterine infections compared to normal cyclic ewes indicate decreased antioxidant capability of these ewes leading to more production of MDA. This decrease may be due to in appetence and especially decrease intake of proteins leading to the depressed immune system [55]. Ascorbic acid (Vitamin C), a multifunctional antioxidant was reported earlier in follicular fluid, ovarian tissue and corpus luteum (CL) of buffalo [56, 19]. Increased serum ascorbic acid recorded in the normal cyclic ewes in comparison to acyclic normal ewes in the present study might be attributed to their role during follicular growth and development as it is required in collagen biosynthesis, which is essential for basement membrane construction during follicle growth (Pinnell, 1985) [57]. The higher serum cholesterol concentration in LN group than FN indicated its important role as a precursor of steroid hormone as in the luteal phase more progesterone needs to be produced by the corpus luteum. Low follicular and serum cholesterol concentration recorded in the FN group might be attributed to the low Mn concentration found in these ewes in the current study. Blood cholesterol being the precursor molecule for steroid biosynthesis, plays a significant role in the synthesis of progesterone, changes in its quantity and type may influence steroid synthesis by ovary [58]. Cholesterol and its fractions get transudated mainly from the serum into the follicular compartment through the "blood-follicle barrier "and the contribution by thecal and granulosa cells through *de novo* synthesis are minimal [59]. In contrary to the report of [17] in the endometritic buffalo follicular fluid, an increased follicular cholesterol concentration in the infectious cyclic group of ewes recorded in the present study might be due to the non-utilization, rather failure in the mechanism of local steroid synthesis in the follicular compartment. Similarly, accumulation of total cholesterol and HDL-C in granulosa cells of early atretic follicles indicates that there is supply of substrate for ovarian steroidogenesis, but it is not being utilized, which may be due to lack of the Cyt.P450 side chain cleavage enzyme or any other similar factor [59]. The non-significantly higher serum level of cholesterol in acyclic ewes compared to follicular normal ewes was in accordance with the report of Vhora *et al.* (1995) [60] in crossbred cows during oestrus. Non-significant increased level of cholesterol in anoestrus animals could be due to its non-utilization for production of steroid hormones [61]. The present study may also take support from the report of [62] who reported significantly lower cholesterol concentration in normal as compared to that of repeat breeder buffaloes. The significantly greater cholesterol level observed in the follicular fluid compared to blood serum irrespective of infectious and cyclic status makes it clear that and confirm the findings of [59] regarding the transudation of cholesterol from the serum to the follicular compartment.

The present study recorded significantly higher serum total protein in follicular normal ewes. Similarly, high serum protein levels were recorded in cyclic heifers [63], buffaloes on the day of estrus [64] and cyclic cross bred cows [60] compared to their acyclic counterparts. Low level of total serum protein in anoestrus condition might cause deficiency of certain amino acids required for synthesis of gonadotropins [60]. The present finding of serum total protein concentration is in agreement with the report of [65] who reported that the total protein concentrations were the same or slightly high during follicular phase compared to luteal phase. The significantly lower level of total protein recorded in the ewes under LI group leading to depressed innate immunity as a result of low globulin content and the development of uterine infections as the animals were under the influence of progesterone. The increased total protein in follicular group might help the animals to maintain its innate immunity and thus they are

protected from uterine infection. The significantly higher serum MDA level in FI and LI than AC group of ewes is in agreement with the earlier reports in cattle suffering from infectious uterine diseases [27, 28]. The lipid peroxidation mechanism destroys cell membranes with the release of intracellular components, such as lysosomal enzymes, leading to further tissue damage [26, 27]. The increased blood MDA concentrations in cows affected with endometritis as compared to healthy animals is apparently due to a marked increase in ROS production during development of the inflammatory process [29]. Greater MDA level was also reported in cyclic cows with subclinical endometritis compared to non-endometritic cows [31]. The increased MDA concentration in the serum of ewes with uterine infections compared to healthy animals in the present study was also found in buffalo cows suffering from uterine infection [66] and post-partum cows suffering from clinical metritis and endometritis [30]. Interestingly these ewes showed decreased ascorbic acid concentration in follicular fluid and serum in the present study indicate that inflammatory uterine diseases are associated with enhanced oxidative reactions and reduced antioxidant defense capabilities.

The higher serum Mn in the luteal group of ewes (LN and LI) might be attributed to the highest availability of MN in CL in comparison to other reproductive tissues [67]. It has been reported that the typical parenchymal cell of the fully functional CL of sheep have abundant mitochondria and this organelle is the principal site of manganese uptake [68]. Low serum Mn concentration found in FN group in the current study may be the reason for low follicular and serum cholesterol concentration and the same is reflected in the serum and follicular progesterone concentration in these ewes. Manganese is important as a cofactor in cholesterol synthesis which in turn is necessary for the synthesis of steroids like progesterone, estrogen and testosterone [69]. On the other hand, high serum Mn concentration in the luteal group of ewes (LI and LN) leads to higher cholesterol and progesterone production in these ewes. It is evident from the study that Mn is an important trace element for reproductive function in ewes. The higher serum zinc concentration in cyclic normal ewes (LN and FN) than the acyclic ewes found in this study was in accordance with the report of [70] who reported that mean serum zinc level in cyclic buffaloes was significantly higher ($P<0.05$) than in anoestrus buffaloes. Similarly, low Zn level was reported in anestrus heifers compared to cyclic heifers [71]. The higher Zn level in the normal cyclic ewes (FN and LN) than their corresponding infectious cyclic group of ewes (FI and LI) indicates its role on the uterine defence mechanism and normal reproductive functions - as supplementation of zinc oxide to Zn plays important role in repair and maintenance of uterine lining following parturition and early return to normal reproductive function and estrus [72]. Higher Zn level was also reported in healthy compared to endometritic buffaloes [66]. Zinc deficiencies have been associated with abortion, fetal mummification, lower birth weight and prolonged labour as Zn plays important role in uterine lining [72]. It is evident from the study that Zn is most important for normal reproductive functions as its level was found high in cyclic normal ewes.

5. Conclusions

Based on the findings of the present study, the following conclusions could be drawn: Higher percentage of neutrophils and TLC in the infectious group of ewes indicates its role in the development of uterine diseases**.** Follicular Nitric oxide concentration was significantly higher in the infectious cyclic group of ewes than the normal ewes. The higher Manganese and Cholesterol concentration in luteal group of ewes indicates its possible role in the synthesis of steroids during luteal stage leading to increased Progesterone production in serum, follicular fluid corpus luteum. The higher MDA level and lower Ascorbic acid concentration in the infectious group of ewes reveals stress in the animals suffering from uterine infections.

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