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Surya Prakash Pannu
Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Govind Narayan Purohit
Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Devendra Prasad Pateer
Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Corresponding Author:
Surya Prakash Pannu
Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Uterine luminal fluid proteins in goats during various reproductive stages (Follicular stage, luteal stage, and early pregnancy)

Surya Prakash Pannu, Govind Narayan Purohit and Devendra Prasad Pateer

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Abstract
The purpose of this study was to assess the changes in uterine luminal fluid proteins during various reproductive stages using goat genitalia obtained from a slaughterhouse. Three groups of genital tracts were classified: early pregnancy (n = 13), luteal phase (n = 21), and follicular phase (n = 26). After the genitalia were dissected, Eppendorf tubes were used to gather samples of uterine fluid. The uterine fluid samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis examination. The molecular weights of the protein bands were determined by comparing their migration rates on the gel with those of protein markers that had known molecular weights. Molecular weights of 198.15, 168.27, 149.64, 118.83, 75.26, 50.33, 46.09, 37.69, 32.96, 21.05, and 15.76 kilodaltons (kDa) were determined by comparing these fractions with those of the standards. Proteins having molecular weights of 46.09 kilodalton were only found in the follicular phase. All reproductive stages showed the presence of proteins with molecular weights of 50.33, 37.69, and 32.96 kDa; however, the greater amounts were noted in the early stages of pregnancy. During early pregnancy, 15.76 kDa fraction of proteins was seen. In both the luteal and follicular phases, proteins with molecular weights of 168.27, 149.64, 118.83, and 75.26 kDa were detected; however, the greater amounts were noted in the follicular phase. All reproductive stages showed the presence of a protein with a molecular weight of 198.15 kDa, although it was unique to the follicular phase. It was concluded that, a greater proportion of low molecular weight proteins develop during early pregnancy and a majority of high molecular weight proteins appear during the follicular phase in the uterus of goats.

Keywords: Goat, uterine luminal fluid proteins, follicular phase, pregnancy, luteal phase

Introduction
The proteins in the uterine luminal fluid change during reproductive cycles and gestations of several mammalian species. The changes in the uterine luminal fluid proteins (ULFP) appear to be necessary act as protease inhibitors (Fazleabas *et al.*, 1982) [10] enzymes (Hansen *et al.*, 1985) [21] carrier molecules for hormone, vitamin, and mineral (Zavy *et al.*, 1979: Buchi *et al.*, 1982: Pentacost and Tang, 1987) [43, 7, 34] pregnancy recognition signalling molecules (Godkin *et al.*, 1984; Bartol *et al.*, 1985; Godkin *et al.*, 1988) [17, 34, 18] immunoregulatory molecules (Murray *et al.*, 1978) [31] as well as several more likely unidentified roles. Some ULFPs are synthesized from the uterine endometrium, while the majority are sequestered from the blood into the uterine lumen (Fischer and Beier, 1986) [11]. The significant variations in the ULFPs have been seen in mares during the estrous cycle (Zavy *et al.*, 1978; Zavy *et al.*, 1979) [43, 44], pigs (Kayser *et al.*, 2006) [25], buffaloes (Kumar and Purohit, 2018) [26], cows (Alavi-Shoushtari *et al.*, 2006; Alavi-Shoushtari *et al.*, 2014) [3, 2]. These modifications are most likely the result of the changed steroids that are in circulation.
It is well known that progesterone in the blood has an impact on the production of uterine proteins (Chen *et al.*, 1975; Adams *et al.*, 1981; Simmen *et al.*, 1991; Trout *et al.*, 1992) [8, 1, 38, 40]. During the follicular and luteal phases of the estrous cycle, progesterone receptors are known to be up or down regulated (Geisert *et al.*, 1994) [14]. The rapid release of histotrophs from the endometrial epithelium that happens between Days 10 and 11 of conception is most likely also caused by fluctuating steroid concentrations (Geisert *et al.*, 1982) [15]. The angiogenesis (Kaczmarek *et al.*, 2010) [24], apoptosis (Ziecik *et al.*, 2011) [45], and extracellular matrix (ECM) remodelling (Diao *et al.*, 2011) [19] most likely change the shape and secretory activity of the endometrium.

When compared to the follicular phase, the uterine glandular epithelium proliferates significantly more during the luteal phase (Grey *et al.*, 2001; Hettinger *et al.*, 2001) [19, 22] Interferon tau is secreted by the trophoblast of a viable embryo in the uterine lumen of ruminants (Bazer, 2013) [6], signalling the release of several proteins in the lumen to aid in the maternal recognition of pregnancy and the preparation of the uterine endometrium for implantation (Forde *et al.*, 2011) [12].

An embryo that is viable stimulates the release of several proteins. The most notable of them was a very basic 14 kDa protein that was created in a transitory manner between Day 15 and Day 24 of pregnancy. Two main proteins are secreted by sheep blastocysts between Days 12 and 21 of pregnancy. According to Godkin et al. (1982) [16] and Masters et al. (1982) [28], one is a large glycoprotein and the other is a low molecular weight protein. There are reports on caprine IFNτ (Lngmarsson *et al.*, 1979; Martinod *et al.*, 1991) [23, 27], and there have been reports on the alterations in cytokeratins and extracellular matrix components in goat endometrium after implantation (Guillomot, 1999) [20] On the other hand, little information is known about the caprine ULFPs during various phases of reproduction. For this reason, the ULFPs in goats at various phases of reproduction were investigated in the current study.

Materials and Methods

Collection of samples

The current investigation was conducted at Bikaner using goat genitalia obtained from abattoirs. Sixty genital organs in all, at the appropriate stage and free of any obvious abnormality, were processed further. Immediately following slaughter, genitalia were and transported to the laboratory in an ice-packed thermocol box.

Screening criteria for selection of genital organs

After examination in the lab, the obtained genitalia were categorised as either early pregnancy or the follicular/luteal stage. There were three further luteal stage classifications: early, mid, and late. Proestrous and estrous genitalia were a part of the follicular phase. The presence or absence of corpus luteum (CL), as well as the colour, size, and consistency of the corpus luteum and the presence or absence of follicles on the surface of the ovary, were used to determine the stage of the estrous cycle (follicular/luteal), as previously described (Roy *et al.*, 2006; Miranda-Moura *et al.*, 2010) [36, 29].

Based on the shape of the corpus luteum, the tracts were categorised as early-luteal (days 1-4, day 1: ovulation), mid-luteal (days 5–10), late-luteal (days 11–16), and follicular phase (day 17–20). In addition to having regressed CL with no vasculature, a cream colour, and a hard texture on the cut surface, the follicular phase also included at least one follicle with a diameter of 5 mm or more. Early luteal stage was defined by ovulation point not covered by surface epithelium, lobular structure of corpus luteum (CL) tissues, and disorganised red colour of the genitalia. Mid luteal stage genitalia featured soft, blood-vessel-filled CL tissues that covered the ovulation site and had an incomplete folding pattern on their cut, reddish-brown surface. The characteristics of late luteal stage, brown CL colour, full folding on the cut surface with a somewhat firm texture, and surface vascularization. The luteal stage encompassed all luteal stages. As previously indicated for cattle, a conceptus that was visible in the uterus during the late luteal phase was believed to be early in pregnancy. (Forde & colleagues, 2014) [13].

Collection of uterine luminal fluid

The excision of the uterine horns were used to get the uterine fluid, which was then carefully scraped from the endometrium using a curette and collected in a 2 mL Eppendorf tube as previously stated (Alavi-Shoustari *et al.*, 2006; Kumar and Purohit, 2018) [3, 26]. A total 2 ml uterine fluid was collected.

Experimental procedure

The three categories of organs were identified: luteal, follicular, and early pregnant animal organs. Eppendorf tubes were used to collect the uterine luminal fluid, which was then processed right away for the SDS-PAGE examination of the proteins in the uterine lumen.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The uterine luminal fluid was processed using commercially available kits (Hi-Media, India) with certain modifications in accordance with the previously established approach (Nandi and Lewis, 1970; Kumar and Purohit, 2018) [32, 26] for the qualitative assessment of proteins.

Analysis of results

After using Gel-doc to capture a picture of the destained gel and reading it on a computer, the findings were computed by comparing the bright band with the protein ladder. With the use of the excel sheet and Image J programme, the band travel distance of the samples and the molecular weights of the proteins under investigation were determined. Using Image-J software, we first determined the migration distance of the known molecular weight marker. Next, we determined the log value of the known molecular weight marker on an Excel sheet.

Using the formula log10 (known molecular weight), the log value of the known molecular weight marker was computed in an Excel sheet.

To get the m and b values (m and b are the absolute cells), we plotted the scatter curve between the log value and the migration distance of the known molecular weight marker. We compute the scatter curve slope value in the Excel sheet using absolute cells, which is y = -0.182x + 5.570. For this value, b = 5.570 and m = -0.182.

With the use of an excel spreadsheet and formula, the migration distance (in centimetres), log value, and molecular weight (in kilo-daltons) of protein bands collected in SDS-PAGE at various reproductive stages were determined.

In order to compute: log value = absolute cell (m) × migration distance throughout various stages of reproduction + absolute cell (b).

Molecular weight (kDa) = 10^log value at various reproductive stages.

Results

Based on molecular weights determined by comparison with a standard ladder on a SDS-PAGE, the uterine luminal proteins were categorised (26 in the follicular phase, 21 in the luteal phase, and 13 in the early pregnancy) (Figure 1). In this work, proteins ranging in molecular weight from 11

kDa to 245 kDa were assessed. The migration distance of the known molecular weight markers (11kDa -245kDa) varied from 1.02 cm to 8.04 cm on the ladder. (Table 1)

Table 1: Migration distance of the known molecular weight markers

S. No.	Known molecular weight (kDa)	Migration distance of known molecular weight (cm)
1.	245	1.02
2.	180	1.70
3.	135	2.20
4.	100	2.90
5.	75	3.90
6.	63	4.60
7.	48	5.10
8.	35	5.70
9.	25	6.30
10.	20	6.70
11.	17	7.60
12.	11	8.04

The formula previously presented was described to determine the log value of molecular weight indicators that are known. The markers on the ladder have log values ranging from 4.041 to 5.389. (Table 2)

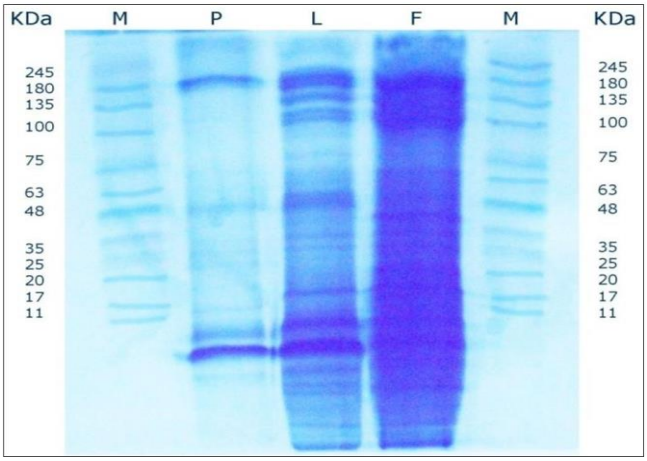

Fig 1: Distained gel showing molecular bands fractions during different phase in SDS- PAGE (Legends: M- ladder, F- follicular phase, L-luteal phase, P-early pregnancy)

Table 2: Log value of known molecular weight markers

S. No.	Known molecular weight (kDa)	Log value of known molecular weight
1.	245	5.389166084
2.	180	5.255272505
3.	135	5.130333768
4.	100	5
5.	75	4.875061263
6.	63	4.799340549
7.	48	4.681241237
8.	35	4.544068044
9.	25	4.397940009
10.	20	4.301029996
11.	17	4.230448921
12.	11	4.041392685

The known molecular weight marker's migration distance and log value were plotted on a scattering curve. (Fig. 2)
To determine the values of m and b, we draw the scatter curve between the log value and the migration distance of the known molecular weight marker (m and b are the absolute cells). We compute the scatter curve slope value in the Excel sheet using absolute cells, which is y = -0.182x + 5.570. For this value, b = 5.570 and m = -0.182.
With the use of an excel spreadsheet and formula, the migration distance (in centimeters), log value, and molecular weight (in kilo-daltons) of protein bands collected in SDS-PAGE at various reproductive stages were determined.
Log value is the sum of absolute cell (m) + absolute cell (b) and the migration distance in each reproductive stage.
Weight of molecules (kDa) = 10^log value at various stages of reproduction

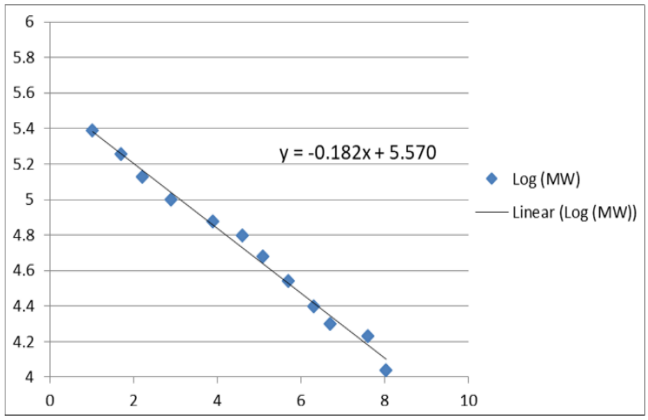

Fig 2: Scatter curve between the log value and the migration distance of the known molecular weight marker

Proteins in uterine fluid during the follicular phase of the estrus cycle

Samples from the proestrus and estrus phases are referred to as follicular phases. During the follicular phase of the estrous cycle, the percentage of protein bands with the molecular weights of 198.15, 168.27, 149.64, 118.83, 75.26, 50.33, 46.09, 37.69, 32.96, and 21.05 kDa was 100, 100, 80.76, 73.07, 26.92, 11.53, 15.38, 19.28, 3.84, and 3.84%, respectively. Estrus-specific high molecular weight proteins were identified. High molecular weight proteins were found in samples of follicular stage, although low molecular weight proteins were also found in a small number of samples.

Proteins in uterine fluid during the luteal phase of the estrus cycle

The proportion of protein bands with their molecular weight 198.15, 168.27, 149.64, 118.83, 75.26, 50.33, 37.69, 32.96 and 21.05 kDa was 90.47, 84.21, 68.42, 57.89, 36.84, 26.31, 31.57, 15.78 and 10.52% respectively during the luteal phase. Low molecular weight proteins were obtained during the luteal phase but also appear during the follicular phase. In the luteal phase, a protein with a molecular weight of 46.09 kilodalton was absent. In the current study, high molecular weight protein bands were also detected during the luteal phase of the estrus cycle. Uterine fluid proteins during luteal phase of estrus cycle

Early-stage pregnancy uterine fluid proteins

During the early stages of pregnancy, the percentage of protein bands with molecular weights of 198.15, 50.33, 37.69, 32.96, and 15.76 kDa was 15.38, 84.61, 100, 100, and 100%, respectively. Lower molecular weight proteins were mostly detected during early pregnancy. During early

pregnancy, a high molecular weight protein band (198.15 kDa) and a protein with a molecular weight of 15.76 kilodalton were detected. Low molecular weight proteins were found in all samples of early pregnancy, although high molecular weight proteins were also found in a small number of samples.

Discussion

The proteins having a molecular weight of 46.09 kDa were discovered in the current investigation only during the follicular phases when the plasma concentration of oestrogens was high. Vascular permeability factor (VPF) is a 40-45 kDa disulfide-linked, homodimeric, heparin-binding glycoprotein that promotes endothelial cell growth and appears to be a good candidate for mediating the increase in vascular permeability and blood vessel growth induced by estrogens in the uterus (Murphy and Ballejo, 1994) [30]. Estrus-specific high molecular weight proteins were identified. The majority of the 198.15 and 168.27 kDa fractions that were discovered during the follicular phase are most likely produced as a result of the estrogenic effect. In their study of buffalo ULFP, Kumar and Purohit (2018) [26] found that during proestrus and estrus (the follicular phase), proteins with mean molecular weights of 207.28±6.65 and 160.28±2.53 were present. According to studies conducted on sheep, certain proteins become more prevalent throughout the follicular phase, and these changed secretory patterns control several aspects of the reproductive process (Soleilhavoap *et al.*, 2016) [39]. The method used in this investigation to extract ULF was gentle endometrial scraping using a curette, which was thought to be more acceptable in other studies (Alavi-Shoushtari *et al.*, 2006; Kumar and Purohit, 2018) [3, 26].

According to the current study on goats, there are more 21.06 kDa protein bands during the luteal phase than during the follicular phase. This finding is consistent with earlier research on buffalo (Roy *et al.*, 2006; Kumar and Purohit, 2018) [36, 26]. In the present study 32.96 kDa protein bands were higher during early pregnancy and lower in luteal phase and follicular phase. A basic progesterone-induced glycoprotein (32 kDa molecular weight polypeptide) was extracted from the uterine secretions of female pigs who had had their ovaries removed in a study conducted by Schlosnagle et al. (1974) [37]. A 15.76 kDa protein bands were only seen in the early stages of pregnancy in our investigation. A research conducted on caprine endometrial tissues by Weise et al. (1993) [42] found that the most significant extremely basic 14 kDa protein(s) were present. These proteins were synthesized transiently between Days 15 and 24 of pregnancy. During this time frame, two more acidic proteins with molecular weights of 14 kDa and 15 kDa were also linked. In sheep (Vallet *et al.*, 1988) [41] and goats (Newton *et al.*, 1996) [33], intrauterine infusion of conceptus-derived proteins or recombinant IFN-τ from days 14 to 18 of the estrous cycle prolongs the luteal life span until around Day 28. Nonetheless, luteal function is preserved despite a significant loss of embryos (Ayalon, 1978) [4]. These losses happen at a point in the pregnancy when the uterine and embryonic tissues are intimately apposed, cellular adhesions are beginning to develop, and the trophoblast and uterine epithelium microvillous interdigitate to establish a final connection that ends in placentation. The developmentally controlled alterations in the apical plasma membranes of uterine luminal epithelial (ULE) and trophoblast cells are thought to promote cellular interactions leading to attachment and beginning of placentation (Powell *et al.*, 2000) [35].

Conclusion

It was determined that, a greater proportion of low molecular weight proteins develop during early pregnancy and a majority of high molecular weight proteins appear during the follicular phase in the uterus of goats.

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Conflict of Interest

The authors have no conflict of interest.

Authors Contribution

Part of research work carried out by Surya Prakash Pannu under the guidance of Prof. Govind Narayan Purohit.

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