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ORIGINAL ARTICLE

Physicochemical characteristics and protein profile of oviductal and uterine fluids from domestic sheep

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ABSTRACT. Oviductal (OF) and uterine (UF) fluids are a complex mixture of ions and macromolecules dissolved in water, derived from the secretions of secretory cells and transudates of the circulatory system. Through proteomics, OF and UF have been analyzed in different domestic species throughout the estrous cycle or during the first days of pregnancy. Therefore, the aim of this study was to evaluate the volume, osmolarity, concentration and distribution pattern of proteins, as well as the identification of OVGP1, HSP70 and ezrin proteins for their importance in reproductive physiology, in OF and UF from adult criollo type domestic sheep during the early luteal phase of the estrous cycle. An average of 3.2±1.5 µL OF and 17±0.5 µL UF per reproductive system were obtained; osmolarity was 343±20.8 mOsm kg^{-1} and 280±96.2 mOsm kg^{-1} and protein concentration was 71.9±23.8 g L^{-1} and 21.8±1.1 g L^{-1}, respectively. In the protein distribution pattern, 20 bands were observed in the OF and 14 bands in the UF. Of these, 14 and 8 were specific for OF and UF, respectively, and 6 were common for both. The spectra of the protein molecular weights were 24–324 and 29–353 kDa for OF and UF, respectively. The presence of OVPG1, HSP70 and Ezrin proteins in both fluids was identified, being in greater quantity in the OF ($P < 0.0005$). The volume recovered from the UF was five times greater than that of the OF. Both osmolarity and protein concentration were higher in OF than in UF (1.2 and 3 times higher). The pattern of protein distribution between the OF and UF was different, being more complex in the OF. OVGP1, HSP70 and ezrin were identified in the OF and UF, and were found in greater quantities in the OF.

Keywords: OVPG1, HSP70, reproductive fluids, osmolarity, SDS-PAGE.

INTRODUCTION

The oviduct is divided into five anatomical regions: infundibulum, ampulla, isthmus-ampullar junction, isthmus, and uterus-tubal junction. Each region participates in gamete maturation, sperm capacitation, fertilization, and the beginning of preimplantation embryonic development (Kölle *et al*., 2020), which ends in the uterus and leads to the implantation process and the beginning of fetal development (Bhusane *et al*., 2016).

The oviductal epithelium and luminal and glandular epithelia from the endometrium are responsible for regulating the microenvironment of each oviductal segment and uterus, formed by populations of secretory and ciliary cells, whose proportion and function depend on the stage of the female estrous cycle or pregnancy (Restall, 1966a; Bhusane *et al*., 2016). Secretory cells synthesize products that are discharged into the lumen and, together with the transudate of the circulatory system, form the oviductal (OF) and uterine fluids (UF), complex mixtures of ions and macromolecules dissolved in water (Bhusane *et al*., 2016; Li & Winuthayanon, 2017).

Sheep embryos remain in the oviduct for an average of three days from the developmental stage of the zygote to the 8 and 16-cells stage. In the uterus, they reach the blastocyst stage by days 6 and 7, the zona pellucida hatching occurs on days 8 and 9 (Abecia & Forcada, 2010), and elongation occurs until day 15. At this point, implantation of the conceptus occurs (Hyttel *et al*., 2010). All of these processes can only be achieved in an environment where nutrients are produced by each structure of the reproductive tract.

Preimplantation embryo development concerning the stages of zygote to blastocyst development can be reproduced under *in vitro* conditions. This requires the use of

synthetic culture medium for embryo development, formulated based on the biochemical composition of the oviductal and uterine fluid (Tervit *et al.*, 1972), which corresponds to the early luteal phase of the estrous cycle (Restall & Wales, 1966; Restall, 1966b; Wales, 1973).

Although this type of medium has been widely used, it is known that embryos produced *in vitro* develop in a minor percentage and quality compared to those produced *in vivo*, and they present dysregulation in their genetic expression and an altered pattern of epigenetic marks (Rabaglino *et al.*, 2021). Therefore, this condition can be reversed when OF and UF are added to the culture medium (Barrera *et al.*, 2017; Canovas *et al.*, 2017; Lopera-Vasquez *et al.*, 2017; Hamdi *et al.*, 2018) as supplements that can supply molecules needed for the adequate development of embryos, such as certain proteins.

Proteomics has been used to describe all proteins expressed in a genome (Wilkins *et al.*, 1996), by which proteins from different organisms have been identified, quantified, and separated through techniques ranging from western blotting and ELISA to more sophisticated and complex techniques such as chromatography and mass spectrometry (Aslam *et al.*, 2017).

The proteome of reproductive fluids has been analyzed in different domestic species, focusing mainly on hormonal regulation at different stages of the estrous cycle, gamete-maternal interaction, embryonic-maternal communication, follicular development, and fertility (Itze-Mayrhofer & Brem, 2020).

However, in the sheep there are few studies related to the proteome of OF and UF, of which most have focused on determining the main proteins present in UF during the first days of pregnancy, but few have reported about OF (Köch *et al.*, 2010; Burns *et al.*, 2014; Romero *et al.* 2017). Only one study has determined the proteome of the cervical, oviductal, and uterine fluids during the estrous and late luteal phases of the estrous cycle in sheep, whether spontaneous or induced (Soleilhavoup *et al.*, 2016). This study identified the main proteins present in OF and UF, including oviduct-specific glycoprotein (OVGP1), heat shock protein 70 (HSP70), and ezrin. Therefore, studies of proteomes in the early luteal phase could be of great interest as early embryo development occurs.

Thus, the objective of the present study was to determine the physicochemical characteristics (volume, osmolarity, and total protein concentration) and protein profile (distribution pattern of proteins) of oviductal and uterine fluids from domestic sheep, as well as to identify OVGP1, HSP70, and ezrin in OF and UF, in the early and middle luteal phases of the estrous cycle in sheep.

MATERIAL AND METHODS

Collecting of OF and UF

OF and UF were obtained from the reproductive systems of adult criollo-type domestic slaughtered sheep. The reproductive systems were obtained immediately after opening the abdominal cavity and were transported at 8 °C in disinfected plastic bags. Once in the laboratory, within no more than 2 h, the reproductive systems were washed in sterile Dulbecco's phosphate-buffered saline (DPBS) with 1% antibiotic-antifungal (streptomycin 10,000 µg mL^{-1}, Amphotericin B 25 µg mL^{-1}, and 10,000 IU penicillin, *in vitro* S.A. de C.V.) at 4 °C.

Subsequently, they were classified based on the ovarian structures present (to determine the stage of the estrous cycle) (Senger, 2005) with regular size and shape. The luteal phase was divided into the early and middle luteal phases. The early luteal phase was determined by the presence of corpus hemorrhagicum, whereas the middle luteal phase was determined by the presence of corpus luteum (Senger, 2005). Thirty-two reproductive systems were in the early luteal phase and ten in the middle luteal phase.

Fluids were recovered from the ipsilateral oviduct and uterine horn of the ovary in the presence of corpus hemorrhagicum and corpus luteum. Once the reproductive systems were classified, the suspensory ligaments were dissected and the uterus-tubal junction was severed to separate the oviduct from the uterine horn. Oviducts and uterine horns were kept in sterile DPBS at 4 °C.

The method described for cattle was followed for OF extraction (Carrasco *et al.*, 2008), with some modifications. On a flat surface, the oviduct was placed and squeezed with a slide from the uterus-tubal junction toward the ampulla, and a clamp was placed to prevent the return of fluid. A fire-polished Pasteur pipette was then introduced to recover the OF and was deposited in a 300 µL tube. The sample was centrifuged at 2000 x g for 5 min at 4 °C, and the supernatant was recovered and centrifuged at 7000 x g for 10 min at 4 °C.

Finally, the supernatant was recovered and stored at -80 °C, until use. To obtain the UF, the reproductive system was placed vertically so that the fluid descended by gravity to the uterus-tubal junction, after which a polished Pasteur pipette was introduced, and the fluid was collected. It was placed in a 300 µL tube, processed, and stored in the same way as the OF. For each experiment, a pull of OF from the early luteal phase and a pull of UF from the middle luteal phase were applied (Carrasco *et al.*, 2008). Subsequently, the volume of each fluid was determined. The OF and UF samples were collected from January to May 2022 (winter-spring), which corresponds to the reproductive season in sheep, and then stored for 8 months at -80 °C. After thawing, the physicochemical characteristics were determined for each fluid (osmolarity, total protein concentration, and OVGP1, HSP70, Ezrin proteins).

Determination of physicochemical characteristics of OF and UF

Osmolarity. Eight months after the samples were collected, the physicochemical characteristics of OF and UF were determined. Osmolarity was determined by placing 10 µL of each sample (OF and UF) in an osmometer (Wescor model VAPRO-5520) (Pensyl & Benjamin, 1999).

Total protein. Total protein concentration was determined using the Bradford method. A calibration curve with bovine serum albumin (BSA) was used as a protein standard with a range of 0 to 1 mg mL^{-1}, 500 µL of Bradford solution (100 mg of Coomassie blue G-250 dissolved in 200 mL of 95% alcohol and 85% phosphoric acid), and 10 µL of each BSA concentration in a 1.5 mL tube, in duplicate. To analyze OF and UF, 500 µL of Bradford solution and 1 µL OF or UF were placed in 1.5 mL tubes, in duplicate. Finally, the calibration curve, as well as the OF and UF samples, were analyzed using a spectrophotometer at 595 nm, and the values obtained to determine the total protein concentration of reproductive fluids were recorded (Bradford, 1976).

Distribution pattern of proteins in reproductive fluids (OF and UF)

Protein separation by SDS-PAGE. Protein separation by molecular weight was performed using electrophoresis, and a 10% polyacrylamide gel was prepared. First, the separator gel was prepared using water, tris-hydroxymethyl-aminomethane (TRIS)-HCl (1.5 M, pH 8.8), bis-acrylamide (30%), Sodium Dodecyl Sulfate (SDS, 10%), ammonium persulfate (10%), and tetramethylethylenediamine (TEMED) and allowed to polymerize. The concentrator gel was then added with the same reagents and only the TRIS-HCl concentration was changed (0.5 M, pH 6.8). Buffer Laemmli was placed with 50 mg mL^{-1} of protein from each sample of OF and UF for protein denaturing. The samples were then placed in each well of the polyacrylamide gel, together with a molecular weight marker of 10–250 kDa. The proteins were compacted at 150 V for 15 min and then left to run at 100 V for 180 min (Brunelle & Green, 2014).

Protein identification by molecular weight. After electrophoresis, the gel was stained with a solution of Coomassie blue (0.1% Coomassie blue, 50% methanol, and 10% glacial acetic acid) for 1 h under constant oscillation. Excess stain was removed using a 40% methanol solution for 15 min during oscillation. Several washes with a new methanol solution were performed until bands were visible in the gel (Brunelle & Green, 2014).

Estimation of molecular weight and band intensity. Images of the gels were taken using a photodocumentary. For image processing, Gelanalyzer (version 19.1; Istvan Lazar Jr.) was used to identify the number of bands in each of the OF and UF samples, as well as the estimation of the molecular weight (kDa) of each of the bands.

Identification of proteins in OF and UF

Western blot. To determine the presence of oviduct-specific glycoprotein (OVGP1), heat shock protein 70 (HSP70) and ezrin which are among the most abundant proteins in these fluids (Soleilhavoup *et al.*, 2016), of each of the OF and UF samples, 50 µL mL^{-1} protein was placed in load buffer and incubated at 95 °C for 5 min. Samples were separated on 10% polyacrylamide gels at 120 volts for 60 min. The proteins were then transferred to a PVDF membrane (Millipore, VH00010) at 2.5 A and 25 V for 20 min on a transfer device (Trans-Blot Turbo).

The membrane was blocked with TBS buffer milk solution (0.08 g mL^{-1}) for 1 h, in constant oscillation at room temperature. The membrane was then incubated with the first antibody (Ezrin: Cat. Sc-58758; HSP70: Cat. sc-66048; OVGP1: Cat. sc-377267, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) diluted in a milk solution with TBS (0.05 g mL^{-1}) in constant oscillation all night at 4 °C. The next day, the membrane was washed with TBS Tween-20 and incubated with the secondary antibody (Anti-mouse IgG: Cat. 715-035-150; Jackson ImmunoResearch, West Grove, PA, USA) for 1.5 h oscillating at room temperature. The membrane was then washed with TBS Tween-20. Finally, a mixture of revealing solution (ClarityTM Western ECL Substrate, Bio-Rad) was added, and the membrane was placed in a photo documenter (Kodak Gel Logic 200 Image System), with a 3 min exposure for its development. The program used was the Kodak 1D 3.6 Logic.

ImageJ software was used for image processing. This program measures the intensity of the pixels in an image. This is especially useful in Western Blot images, where the signal intensity can be correlated with the amount of a certain protein. To do this, the program selects the Region of Interest (ROI) around a specific region of the image in which the analysis needs pixel intensity. This generated a graph that shows the pixel intensity of the ROI. ImageJ provides numerical values associated with the ROI, from which statistical analysis will be performed.

Statistical Analysis

The data obtained on volume, osmolarity, and total protein concentration of OF and UF samples are expressed as the mean ± standard deviation (S.D.). To determine significant differences between the color intensities OF and UF sample bands, Student's t-test was performed with a significance of $P < 0.05$. GraphPad Prism (version 9.5.1) was used for data analysis.

RESULTS

OF and UF were obtained from 42 reproductive systems of domestic sheep. Of these, 32 were in the early luteal phase and 10 were in the middle luteal phase. A pool of OF (2-7 reproductive systems) and UF (1-6 reproductive systems) was prepared for each experiment, and a total of 10 OF samples and 3 UF samples were obtained.

Physicochemical characteristics of OF and UF

Volume, osmolarity, and total protein concentration of the reproductive fluids (OF and UF). A total volume of 135 µL (3.2±1.5 µL on average) of OF from the early luteal phase and 170 µL (17.0±0.5 µL) from the middle luteal phase were recovered from the reproductive apparatus at the luteal phases of the estrous cycle in sheep. The osmolarity for OF was 343±20.8 mOsm kg^{-1}, and that for UF was 280±96.2

mOsm kg^{-1}. The total protein concentration of OF was 71.9±23.8 g L^{-1}, while that of UF was 21.8±1.1 g L^{-1}.

Distribution patterns of proteins in the reproductive fluids (OF and UF). Figure 1 shows the pattern of protein distribution in 10 OF and 2 UF samples. The molecular weights of the protein bands were 24–324 kDa for OF and 29–353 kDa for UF.

In the analysis of the OF and UF samples, two sets of bands were identified, with 20 bands present in the OF and 14 bands present in the UF. Of these, fourteen bands were unique to the OF samples, while eight were exclusive to the UF samples. Furthermore, six bands were identified as being present in both the OF and UF samples (as shown in Figure 1 and detailed in Table 1).

Identification of proteins in OF and UF. In the distribution pattern of OF and UF proteins (Figure 1), different types of proteins are shown, some of which were identified by western blotting, where OVGP1 (120 kDa), HSP70 (70 kDa), and ezrin (87 kDa) proteins were present in most OF and UF (Figure 2a). However, the levels of these proteins were significantly higher in the OF than in the UF (Figure 2b).

Table 1.
Molecular weight of proteins present in OF and UF samples.

No. of band	OF specific MW (kDa)	UF specific MW (kDa)	In common MW (kDa)
1	324	353	73
2	315	284	37
3	306	138	34
4	228	111	28
5	200	47	27
6	188	42	26
7	83	39	
8	62	29	
9	54		
10	36		
11	35		
12	33		
13	32		
14	24		

OF, Oviductal fluid; UF, Uterine fluid; MW, Molecular weight; kDa, kiloDaltons

DISCUSSION

Physicochemical characteristics of OF and UF. Two pools were created in this study. One was of OF from the early luteal phase and the other was a pool of UF from the middle luteal phase because embryos are in each one of these structures as luteal phases occur (Kölle *et al*., 2020). OF and UF were collected from *postmortem* adult Criollo type domestic sheep, with a volume of UF five times greater than OF, which may be related to the size of the organs of origin. In other studies, OF and UF volumes were collected from sheep *in vivo* and analyzed continuously for 24 h using a probe introduced into the ostium of the oviduct. The authors reported higher volumes of OF and UF compared to those obtained in our study, with 100 to 1,630 µL for OF (Restall, 1966b; Perkins *et al*., 1965; Iritani *et al*., 1969; Roberts *et al*., 1976) and 480 to 5,210 µL for UF (Iritani *et al*., 1969). These volumes account for the intense secretory activity of the oviduct and uterus, which may be influenced by the probing recovery system, is also influenced by hormonal action. It has been observed that 2 days after estrous, both the oviduct and uterus present greater secretory activity (Iritani *et al*., 1969), which confirms that there are factors related to the modulation of the secretory pattern of the fluids in these anatomical regions. In addition, other factors influence the secreted amount of OF and UF, for example, such as age, breed, and whether the females are prepubertal, nulliparous, or multiparous.

Another important physiological aspect to consider is the osmolarity of OF and UF, which depends on the concentration of dissolved solutes, such as inorganic salts, amino acids, and proteins, among other components, which are in direct contact with gametes and developing embryos that require specific osmolarity conditions. The range of variation of oviductal and uterine osmolarity between different

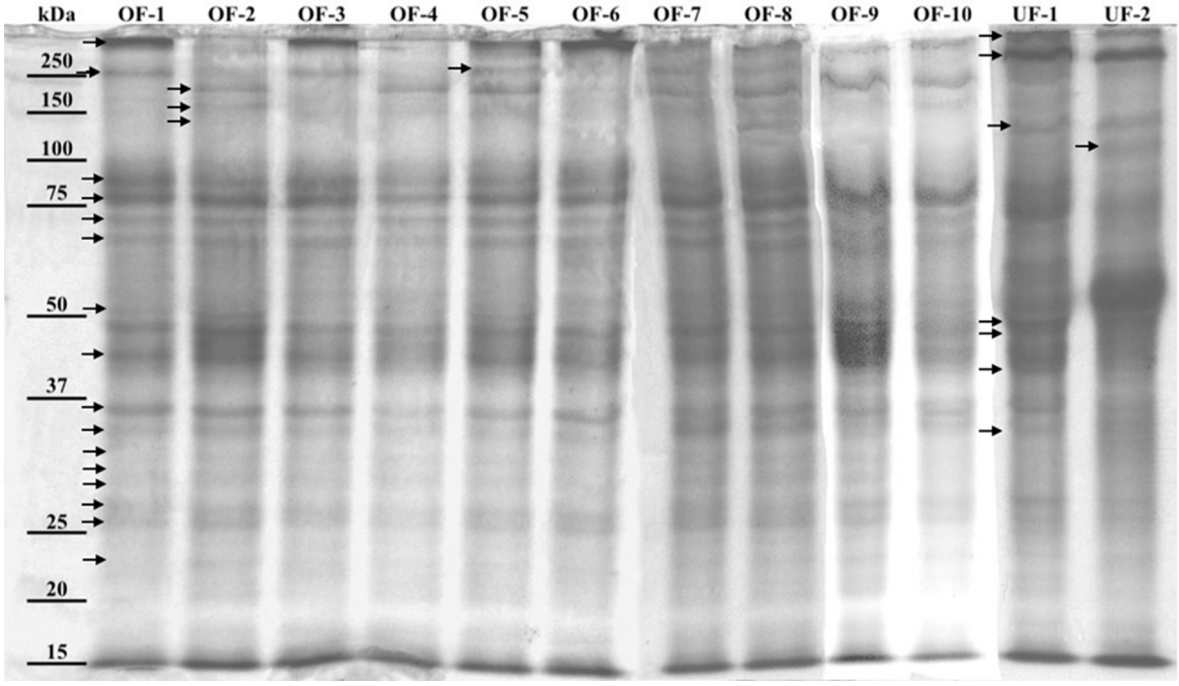

Figure 1.
The allocation of proteins in the sheep oviductal fluids (OF-1 to OF-10) and uterine fluids (UF-1 and UF-2) was assessed through SDS-PAGE. Fourteen bands where unique to OF samples, eight were exclusive to UF samples, and six were present in both samples. Specific molecular weights of proteins are indicated in Table 1.

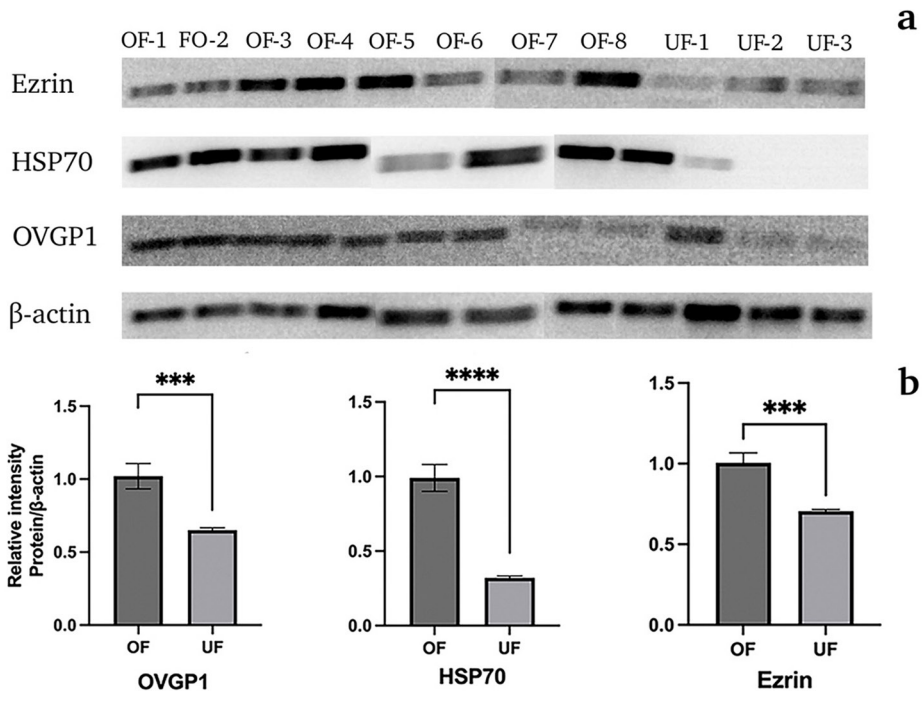

Figure 2.
The presence of OVGP1, HSP70, and ezrin proteins was examined in the OF and UF samples from sheep using Western blotting. β-actin was used as a positive control (a). The concentration of OVGP1, HSP70, and ezrin was then assessed between the OF and UF samples, revealing a statistically significant difference between the two groups (P = 0.0005 and P = 0.0001, respectively) (b).

domestic species is narrow; for example, in cows, an osmolarity of 350–353 mOsm kg^{-1} has been reported (Olds & Vandemark., 1957), in sows it is 318-320 mOsm kg^{-1} (Li *et al*., 2007), and in sheep, an osmolarity of 316-349 mOsm kg^{-1} has been reported for OF and UF (Wales, 1973). These values are similar to those observed in this study for OF (343 mOsm kg^{-1}) but lower than that observed in UF (280 mOsm kg^{-1}). This variation in UF osmolarity was probably due to manipulation during the collection and processing of samples; for example, it has been reported that both the method and collection site influence the osmolarity of UF (Wales, 1973).

The total protein concentration present in OF was higher (71.9 g L^{-1}) than that reported in other studies with concentrations of 37.8 and 38.7 g L^{-1} (Itze-Mayrhofer & Brem, 2020; Zhao *et al*., 2022). The total UF protein concentration was similar (21.8 g L^{-1}) to that reported by other authors (25.16 to 27.46 g L^{-1}) (Tripathi *et al*., 2016; Yahia *et al*., 2013). The increase in total OF protein concentration could be due to metabolic stress conditions derived from the diet (Tripathi *et al*., 2016).

In a first evaluation, the distribution pattern of the proteins present in OF (20 bands) and UF (14 bands) was determined, as well as the molecular weights of the bands with greater intensity, as has been done in Alpaca (Apichela *et al*., 2015). From this first count, differences in the distribution pattern of the proteins present between the OF and UF samples were observed, a greater number of bands for OF being observed, which denotes a greater complexity in their protein composition, However, studies have identified more proteins in UF than in OF (827 vs. 624), a fraction of these proteins (585) occur in both fluids (Soleilhavoup *et al*., 2016).

Identification of proteins in OF and UF. Subsequently, three proteins were identified using western blotting: OVGP1, HSP70, and ezrin. OVGP1 is a protein synthesized and secreted exclusively by the non-ciliary cells of the oviductal epithelium, with a molecular weight between 90 and 95 kDa for most domestic animals; in ruminants, this is in the range of 57.23–57.75 kDa, which is attributed to the degree of glycosylation of the protein (Pradeep *et al*., 2011; Zhao *et al*., 2022).

Moreover, HSP70 belongs to a family of "chaperones" proteins with a molecular weight of 70 kDa that perform a wide variety of cell maintenance activities, as well as to counteract the effects caused by stress, such as: preventing protein aggregation, the separation of protein aggregates, the replication of denatured proteins and the degradation of defective proteins (Rosenzweig *et al*., 2019).

Ezrin is an protein of 87 kDa molecular weight that functions as a microfilament connector of the plasma membrane, which is distributed in the microvilli, folds of the plasma membrane, and other areas of this membrane, with a particular morphology (Xu *et al*., 2023).

In the present study, OVGP1 was found in greater numbers in OF samples, which is in accordance with Soleilhavoup *et al*. (2016), who evaluated the OF and UF proteome in estrous and during the luteal phase of the sheep estrous cycle, by nanoscale liquid chromatography coupled to tandem mass spectrometry. The authors determined that OVGP1 was the main OF protein during estrous. In contrast, OVGP1 was present in smaller quantities in UF, which corresponds to what was reported for UF (Soleilhavoup *et al*., 2016). However, extracellular vesicles obtained from the uterine lumen of sheep have been described as positive for OVGP1 (Burns *et al*., 2014).

OVGP1 plays a significant role during fertilization because it regulates polyspermy by hardening the pellucida zone (Bragança *et al*., 2021). It has also been reported in goats that morulae and blastocysts rate increases (Pradeep *et al*., 2011). Immunohistochemistry has detected the interaction of OVGP1 with bovine embryos in stages of 4-8 cells and in morulae, in the perivitelline space, and within blastomeres, but not in the pellucida zone (Banliat *et al*., 2020).

The presence of HSP70 protein was observed in the OF, but it was practically absent in the UF, which coincides with what has been reported in sheep (Soleilhavoup *et al*., 2016). However, others have identified it in the UF on day 16 of the estrous cycle, but not in sheep on day 16 of pregnancy (Köch *et al*., 2010). Microvesicles of the uterine lumen are positive for HSP70 on day 14 in both cycling and pregnant sheep (Burns *et al*., 2014).

As mentioned, HSP70 performs activities to counteract the effects of stress. Under suboptimal temperatures of *in vitro* culture (37 and 40 °C), HSP70 is overexpressed in both oocytes and granulosa cells (Pöhland *et al*., 2020). This has also been reported in cattle, where inhibition of HSP70 function during culture of 2-cell embryos at physiological temperature (38.5 °C) reduces the percentage of blastocysts. This shows the importance of HSP70 in cellular functions, not only in relation to caloric stress (Romero & Hansen, 2002).

Finally, ezrin protein was found in greater quantities in OF than in UF, which is consistent with the results reported for sheep (Soleilhavoup *et al*., 2016). It has also been reported in the UF of sheep during the first days of pregnancy (Köch *et al*., 2010; Romero *et al*., 2017).

Xu *et al*. (2023) reviewed the function of ezrin protein from a reproductive perspective, mentioning its importance during changes that occur in the uterine epithelium throughout the estrous or menstrual cycle. Ezrin induces polarization of preimplantation embryos as well as processes of cellular migration and invasion during embryo implantation and embryogenesis.

The volume recovered from UF was five times greater than that recovered from OF, which is related to the size and surface area of the oviduct and uterus. Both osmolarity and protein concentration were higher in OF than in UF (1.2 and 3 times higher, respectively), values that were within the range described for sheep. The pattern of protein distribution between the OF and UF was different, being more complex in the OF. OVGP1, HSP70, and ezrin were identified in OF and UF, and were found in greater numbers in OF samples.

CONCLUSIONS

The present study determined the physicochemical characteristics (volume, osmolarity, and total protein concentration), protein profile (distribution pattern of proteins), and the presence of OVGP1, HSP70, and ezrin proteins in the early and middle luteal phases of the estrous cycle of adult domestic sheep to determine its usefulness as a supplement to optimize *in vitro* culture media for embryo development by providing elements lacking commercial growing media.

Competing interest's statement

The authors declare that they have no conflicts of interest.

Author contribution

Conception: D.A.A.G., G.B.F., M.A.F.B. and J.R.V.A. Execution of experiments: J.R.V.A., H.L.R., M.A.F.B., and M.P.C.O. Data analysis and interpretation: M.A.F.B., D.A.A.G., and J.R.V.A. Original draft: J.R.V.A. Revisions: D.A.A.G., C.C.R., G.B.F., M.A.F.B. and M.C.N.M. Disposition of funds, materials, and equipment: D.A.A.G., G.B.F., and M.C.N.M.

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