UTERINE LUMINAL FLUID PROTEIN CONTENT IN VIABLE AND NON-VIABLE *CONCEPTUSES* IN SHEEP

CONTENIDO DE PROTEÍNAS DEL FLUIDO LUMINAL UTERINO EN *CONCEPTUS* OVINOS VIABLES Y NO VIABLES

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ABSTRACT

During implantation, embryo survival depends on the availability of uterine and/or trophoblastic secreted molecules, including proteins. In order to determine possible differences in the protein profile of the uterine luminal fluid (ULF) adjacent to the embryonic site of viable and non-viable *conceptuses* at 20, 28 and 35 days of gestation, two-dimensional (2D) PAGE electrophoresis was carried out. The ULF adjacent to viable *conceptuses*, had greater amounts of proteins as compared to correspondent sites of non-viable *conceptuses*. In all the studied gestational period there were several proteins in the ULF near viable embryos, which were absent in the corresponding areas of the non viable ones. Two of these proteins were identified. One corresponds to transferrin, which can be produced either by the *conceptus* or the mother. It is important for proliferation, differentiation, and cellular function, since it is an iron transporting and binding molecule. Its absence in the ULF near non viable embryos might be interpreted as a lack of production by the *conceptus*. Likewise, transferrin appears to be involved in the formation of blood vessels, which then could account for the absence of those structures in the membranes of non viable *conceptuses*. The other protein corresponds to aldose reductase, which is responsible for sorbitol and fructose synthesis, although its metabolic role during implantation is still unknown. In conclusion, some proteins present near viable embryos are absent in non viable *conceptuses* at days 20; 28 and 35 of gestation which could be indicators to determine, at least partially, embryonic survival during the studied period.

Key words: Proteins, uterine fluid, embryonic mortality, implantation, sheep.

RESUMEN

Durante la implantación, la supervivencia del embrión depende de la habilidad del útero y/o trofoblasto para secretar moléculas, incluyendo proteínas. Para determinar las diferencias posibles en el perfil proteico del fluido luminal uterino (FLU) adyacente al embrión de *conceptus* viables y no viables a los 20, 28 y 35 días de gestación, se realizó electroforesis bidimensional (2D-PAGE-SDS). El FLU adyacente a *conceptus* viables, tuvo mayor cantidad de proteínas al compararlo al correspondiente de *conceptus* no viables. En todos los periodos de gestación estudiados hubo varias proteínas en el FLU cerca de embriones viables, las cuales estuvieron ausentes en la correspondiente área de los embriones no viables. Dos de estas proteínas fueron identificadas. Una de ellas corresponde a la transferrina, la cual puede ser producida, tanto por la madre como por el *conceptus*, y es importante para la proliferación, diferenciación y función celular, debido a su capacidad de unir y transportar hierro. Su ausencia cerca de los embriones no viables es interpretada como una falla en la producción por el *conceptus*. También, la transferrina parece estar involucrada en la formación de vasos sanguíneos, la cual podría ser responsable de la ausencia de esta estructura en las membranas de *conceptus* no viables. La otra proteína encontrada correspondió a la aldosa reductasa, la cual es responsable de la síntesis de fructosa y sorbitol, aunque el papel de este metabolito durante al implantación es desconocido. En conclusión, algunas proteínas presentes cerca de embriones viables estuvieron ausentes en *conceptus* no viables a los días 20; 28 y 35 de gestación, lo cual puede ser un indicador para determinar, al menos, parcialmente, la supervivencia embrionaria durante el periodo estudiado.

Palabras clave: Proteínas, fluido luminar uterino, mortalidad embrionaria, implantación, ovejas.

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INTRODUCTION

Embryonic mortality (EM) is considered the most important cause of prenatal losses in sheep (*Ovis aries*), having an incidence of 36.7% [12]. An important proportion of EM occurs during implantation, when the embryo is particularly labile, since it evolves in a slow and gradual manner. It ensues near the embryo at day 15 of gestation and spreads peripherally thereafter, and it is not complete before day 60 of gestation, when placentomes are well developed [5, 8, 9]. The ovine *conceptus* remains floating within the uterine lumen before its attachment to the uterine endometrium. Its nutrition depends on the small volume of uterine luminal fluid that probably contains all necessary nutrients for the development in this phase, and even after implantation [13].

The embryo depends for its survival, among other factors, on molecules secreted by the uterus, such as growth factors and proteins. They act as growth promoters and immunomodulators [18]. There is extensive work in the identification of proteins secreted by the endometrium, which are controlled by estrogens and progesterone [1, 6, 17]. However, information is lacking on the possible changes in protein secretion with the advancement of gestation during implantation in sheep. Furthermore, it is feasible that the absence of one or more molecules could be crucial for embryonic survival.

Induction of multiple pregnancies by superovulation in sheep, offers reasonably possibilities to obtain both, viable and non-viable *conceptuses* for comparative studies [15].

The present trial was undertaken to characterize the electrophoretic profiles of secreted proteins into the uterine lumen of sheep at 20, 28 and 35 days of gestation in both, viable and non-viable embryos.

MATERIALS AND METHODS

The work was carried out in the correlation of laboratory experimentation and Morphophysiology and Histopathology of the Graduate School of Veterinary Medicine and Zootecnia of the Universidad Nacional de Colombia, Bogotá DC, approx. 2640 m, with an annual average temperature, relative humidity and rainfall of 12°C, 79% and 938 mm / year, respectively [10].

Twelve crossbred sheep, healthy and multiparous (two to five births) randomly allocated into the following groups: a) days 20 (n= 4), b) 28 (n= 4) and, c) 35 (n= 4) of gestation.

The animals were fed with natural pasture (*Pennisetum clandestinum*) using a daily supplement of concentrated commercial feed containing 14% protein, mineralized salt and water *ad libitum*.

Estrous detection was carried out with the assistance of a caudoepididymectomised ram. Daily progesterone levels were assessed by radioimmunoassay (RIA), using commercial kits[1], to verify normal luteal function (average levels of 0.1621 and 4.761 nanograms of P_4 for days 0 and 12, respectively). This test has a sensitivity of 0.02 ng / mL, and a very low cross-reactivity with other components present in the sample. A treatment samples were measured P_4 levels by RIA in different tests. The intra-assay coefficient of variation average was 8.001% and the intra-assay variation of 8.31%.

When animals fulfilled the established gestational age, were anesthesized and then slaughtered by exsanguination.

The initiation of estrus was set as day 0 of the estrous cycle. Sheep were synchronized for estrous using intravaginal sponges, containing 60 mg of medroxyprogesterone acetate[2], for 13 days. Forty eight hours prior to sponge withdrawal, animals received 1500 IU (im) of Pregnant Mare Serum Gonadotropin (PMSG)[3]. Each female was given 2.5 mL (iv) of PMSG[4] antibodies at day 0, as to neutralize possible secondary effects of PMSG. The animals were allowed to have 2 estrous cycles before they were naturally mated.

The reproductive tract was extracted and uterine luminal fluid (ULF) was obtained at site location of each embryo, using a sterile aqueous solution of NaCl (0.15M) for local washes. Protease inhibitors were added to all samples, which were then centrifuged in sterile vials at 10000 rpm for 10 minutes at 4°C (cooling centrifuge. Eppendorf, 5417R, USA). The supernatant was then conserved at -20°C (Freezer Indufrial, BGL-320, Colombia).

Before the analysis, all samples were centrifuged and the sediment placed in culture media for bacterial and fungal cultures. Afterwards, they were stained with Gram's and observed under the light microscope (Olympus microscope CX21, Tokio, Japan) to verify the absence of bacteria, fungi or blood cells.

Protein concentration was then determined using the Bradford´s method [4], with bovine serum albumin (BSA) as the standard. Equal amounts of proteins were employed for two-dimensional -PAGE, which was carried out following the procedure described by O´Farrell [15], using an electrophoresis chamber MINI PROTEAN III, Bio-Rad, USA,. Briefly, up to

1 Diagnostic Products, USA.

2 Depo-Provera, Upjohn, USA.

3 Folligon, Interver, Holland.

4 Neutra PMSG, Intervet, Holland.

10 µg of total protein was loaded onto each prefocused 7.5 cm x 25 mm (i.d.) tube gel containing 1.6% (w:v) ampholytes (pH 5–7) and 0.4 (w:v) ampholytes (pH 3.6–9.5), and proteins were separated by isoelectric focusing. The gels were extruded from the tubes, equilibrated with 62.5 mM Tris-HCl pH 6.8 containing 10% (w:v) glycerol, 5% (v:v) 2-mercaptoethanol, and 2.3% (w:v) sodio dodecilsulfato (SDS), then attached to 0.075 x 7 x 8-cm 11 and 15% (w:v) acrylamide SDS gels, and subjected to electrophoresis in the second dimension. After electrophoresis, proteins were detected by non-ammonium silver staining, a preparation where band staining depends on the union of silver with more than one chemical compound in the protein. The range of detection was 2-5 ng/protein. For analysis, the gels were registered photographically, using a digital camera SONY Mavica, 1.3 Mega Pixels, model MVC-FD88, Canada.

In order to verify pH gradients and validate the standarization for the first dimension, some isoelectric focus gels were cut in 5mm sections and then placed in individual vials, in which 2 mL of a 9.2 M urea degasified aqueous solution had been added. The obtained pH reading for each sample was within the expected range, following O'Farrell's recommendations [15].

Embryonic viability was estimated by the macroscopical characteristics of the embryonic membranes, degree of vascularization of the allantochorion and embryo development, according to that described by Boshier y Guillomot [3, 8].

Descriptive statistics, and correlative studies, using the Minitab Program 7.2 [14]. Multivariate analysis was used, to determine possible differences in protein electrophoretic profiles among variables.

RESULTS AND DISCUSSION

Sixty embryos were found in all studied sheep, with a mean value of 5 embryos/ewe. Thirty six percent of them were classified as non-viable, based on at least 1 of the following macroscopic characteristics: absence of blood vessels in the allantochorion, yellowish colour of embryonic membranes and absence of an embryo.

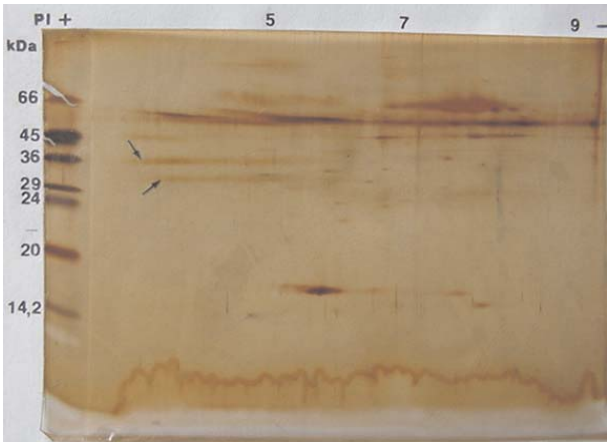
This is the first report of protein content of secretory products adjacent to non viable and viable ovine *conceptuses*.

There were no individual differences in the electrophoretic profile of the embryos in the same condition (viable or nonviable). Hence, for the 2D-PAGE electrophoretic analysis, data were pooled for each condition of the embryos (viable or nonviable) in each age studied. The employed experimental procedures allowed the detection of proteins having 6.5 to 205 kDa, within an isoelectric point ranging from 3.6 to 9.5.

TABLE I
DIFFERENCES IN PROTEIN CONTENTS IN ULF ADJACENT TO VIABLE AND NON-VIABLE EMBRYOS IN SHEEP DURING IMPLANTATION

			Day of Gestation					
			20		28		35	
Prot. # (Spot)	MW (kDa)	Ip	E.V	E.N.V	E.V	E.N.V	E.V	E.N.V
23-25	80	6.8–7.3	✓	-	✓	-	✓	-
36-38	70	6.9-7.1	✓	✓	✓	-	✓	-
75	56.8	5.44	-	-	✓	✓	✓	-
76	56	5.14	-	-	✓	✓	✓	-
96	55	4.7	-	-	-	-	✓	-
97	55	4.9	-	-	-	-	✓	-
132-144	32	3.7-5.1	✓	-	✓	✓	✓	✓
148-149.1	31	6.6-6.8	✓	-	✓	-	✓	-
150	30	8.5	✓	✓	✓	✓	✓	-
151-160	30	3.8-5.0	✓	-	✓	✓	✓	✓
184-190	24-27	7.0-7.9	-	-	✓	✓	✓	-
203	17.03	4.0	-	-	✓	-	-	-
205	16.5	4.1	-	-	✓	-	-	-
208	17.5	5.4	✓	-	✓	✓	✓	✓
215	16	7.3	✓	-	✓	-	✓	-
219	14.9	8.2	✓	-	✓	-	✓	-
223	<6.5	5.0	-	-	✓	-	-	-

MW= Molecular Weigth, Ip= Isoelectric point, EV= Viable embryo, NV= Non viable embryo, √: Present; -: absent.



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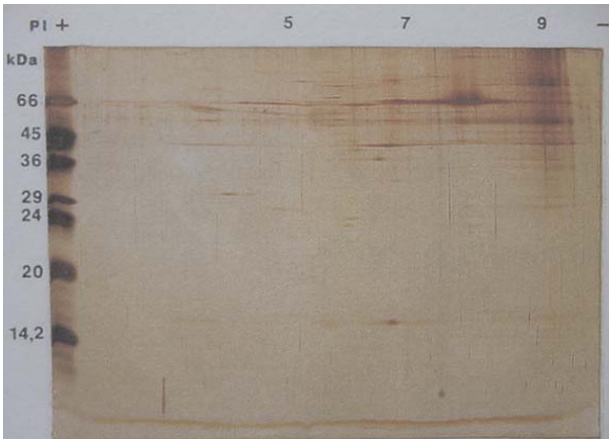

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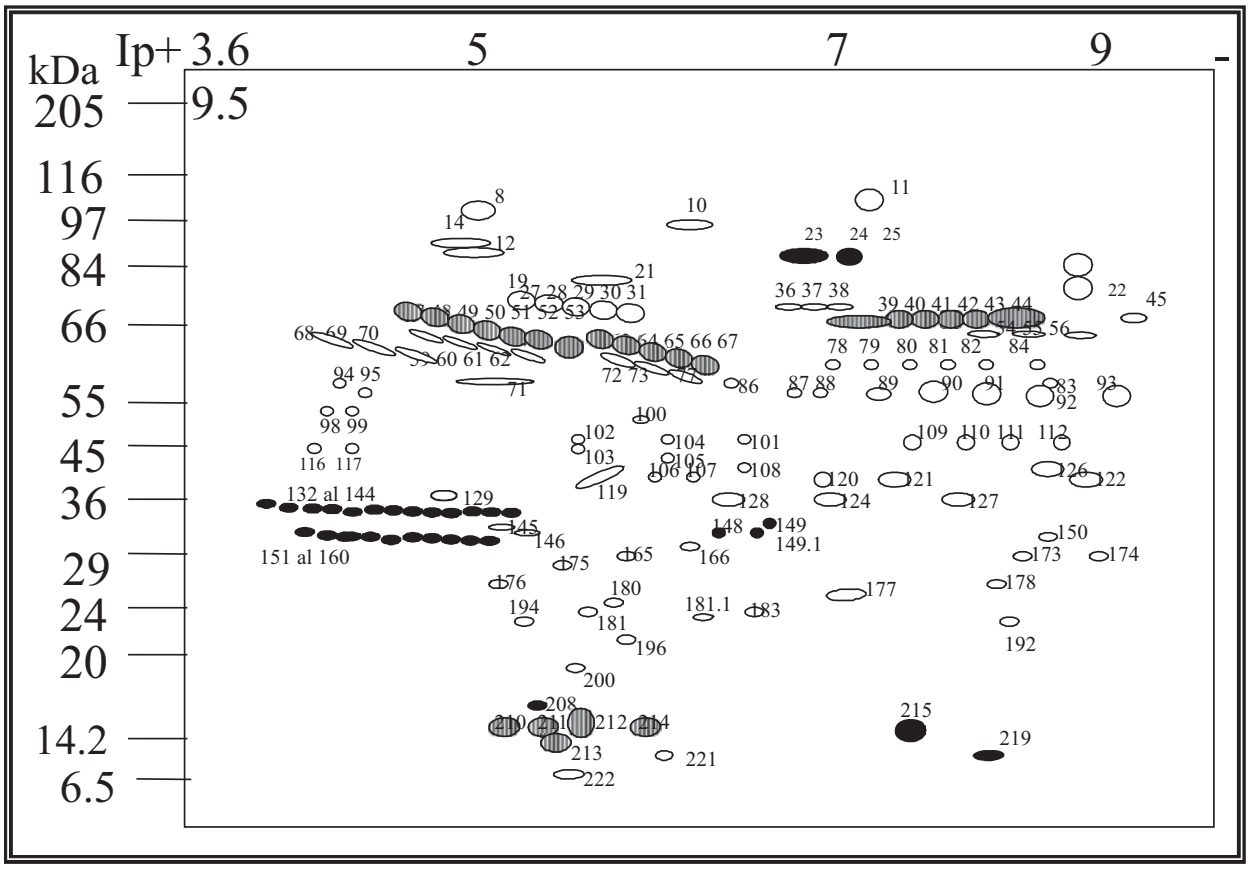

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FIGURE 1. 2D-SDS-PAGE. DETECTED PROTEINS IN THE UTERINE LUMINAL FLUID AT 20 DAYS OF GESTATION, (A) CORRESPONDING TO VIABLE EMBRION'FLU AND (B) NON VIABLE EMBRION. QUALITATIVES DIFFERENCES ARE REPRESENTED BY THE ARROWS. (C) SUMMARY OF THE TOTAL NUMBER OF DETECTED PROTEINS IN THE UTERINE LUMINAL FLUID AT 20 DAYS OF GESTATION. PROTEINS PRESENT IN ALL EMBRYOS ARE SHOWN AS ○ . QUALITATIVE DIFFERENCES, AS ● (ONLY FOUND IN VIABLE EMBRYOS). QUANTITATIVE DIFFERENCES ARE SHOWN AS ◍ (VIABLE EMBRYOS > NON-VIABLE ONES) AT THE LEFT SIDE APPEAR MOLECULAR WEIGHT MARKERS AND THE SEPARATION CHARGE PRODUCED BY ISOELECTROFOCUSING IS SHOWN AT THE TOP OF THE FIGURE. MOLECULAR MASS MARKERS ARE INDICATED ON THE LEFT.

At the embryonic sites of viable embryos, there was a greater amount of proteins in the ULF adjacent to viable embryos as compared to correspondent sites of non-viable *conceptuses*: 35, 19.2 and 21% at 20, 28 and 35 days of gestation, respectively. The quantitative differences encountered were represented by 4 complex groups, namely, protein spots 39-44, 47-52, 63-67, and 210-214, with molecular weight (MW) of 66 kDa with Isoelectric Points (Ip) ranging from 7.18-8.3; 60-58 kDa (pI 4.8-5.6); 59-56 kDa (Ip 5.8-6.4); 14.2 kDa (Ip 5.2-6.2), respectively.

At 20 days of gestation, there were several proteins in the ULF adjacent to viable embryos, which were absent in the correspondent sites of non-viable *conceptuses*. Those molecules were represented by several protein complexes (FIG. 1): 1) MW ~32 kDa with Ip ranging from ~3.7 a 5.15 (protein spots 132 - 144), 2) MW ~30 kDa and Ip ~3.84-5.03 (spots 151 –160), 3) MW ~80 kDa and Ip ~6.8-7.3 (spots 23 –25), those which migrated in a similar position to that of the group of proteins identified by Lee et al. [13], as transferrin in 2D gels of the ovine uterine luminal fluid proteins, and 4) MW ~31 kDa and Ip ~6.6-6.8 (spots 148 – 149.1). The latter protein, did migrated to a similar position to that of a molecule known as aldose reductase, identified by Lee et al. [13], using 2D electrophoresis, this enzyme is responsible for the synthesis of sorbitol and fructose, but its role in implantation is unknown. It is possible that this protein has more unidentified functions [13]. Likewise, non-grouped proteins were found (TABLE I). It is tempting to propose those proteins can be taken as markers for embryonic viability. Some of these proteins corresponding to spots 132-144 and 151-160), might be of maternal origin, since they were also encountered in the uterine luminal fluid of cycling animals (unpublished data). Furthermore, regional differences in protein secretion could be the result of either uterine incompetence to produce certain compounds or, that a viable embryo induces the uterine epithelium to secrete specific proteins. Also, the abovementioned difference could be interpreted as the result of inhibition to uterine secretion, by a non-viable *conceptus*. Some groups of proteins, at day 20 of gestation, were found in both, viable and non-viable *conceptuses*. However, there were a higher amount of them in the viable *conceptuses*, as compared to the non-viable ones (FIG. 1).

Again, at 28 and 35 days of gestation, there were proteins in the ULF adjacent to viable embryos, which were lacking in the correspondent sites of non-viable *conceptuses*. They corresponded to compounds of MW ~32 y 30 kDa (Ip ~3.7-5.15 and ~3.84-5.03, respectively). However, there were differences in the amount of these molecules between the abovementioned gestation-ages (FIG. 2). As observed for 20, 28 and 35 days of gestation, protein spots 215 (~16 kDa, Ip ~7.3), 219 (~14.9 kDa and Ip ~6.2), and 23 – 25 (~80 kDa and Ip ~6.8-7.3) denominated transferrin and protein spots 148 – 149.1 (aldose reductase), were non-detectable in non-viable embryos. The following compounds were found in viable embryos, and absent in the non viable ones at 28 days: protein spots 36-38, 148, 149, 149.1, 200, 203, 205 y 223), with corresponding MW and pI were: ~80 kDa (Ip ~6.8 – 7.3); ~70 kDa (Ip ~6.9-7.1); ~31 kDa (Ip ~6.2); ~31 kDa (Ip ~6.8); ~30.5 kDa (Ip ~6.6); ~18.5 kDa (Ip ~5.7); ~17.03 kDa (Ip ~4.0); ~18.5 kDa (Ip ~4.1); and <6.5 kDa (Ip ~5.01), respectively (TABLE I; FIG. 2). At day 35, additional qualitative differences were represented by the absence in non-viable embryos of two protein complexes: one, with MW ~27-24 kDa (Ip ~7.0-7.9, spots 184-190). Non-grouped proteins were also found (TABLE I; FIG. 3). Therefore, it could also be thought that differences in protein expression after 20 days might be taken as indicative of embryonic decay.

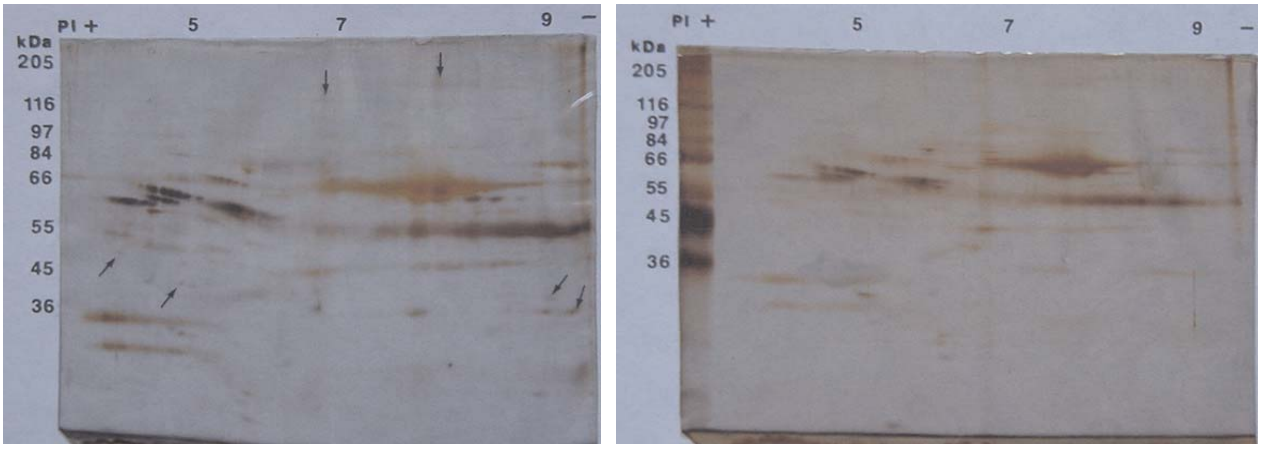
At all gestational ages studied, spots 215 and 219 (MW 16kDa (Ip 7.3) and 14.9 kDa (Ip 8.2), respectively were absent in non-viable *conceptuses*, but present in viable ones. Nevertheless, there are no reports about proteins in ULF with a similar pattern of migration in two dimension gels, as presently found.

An important difference in the three studied gestation ages was the presence, only in viable embryos, of a protein which migration in 2D gels coincides with the physical characteristics of transferrin (protein spots 23-25), shown to derive from both the maternal and the *conceptus* side; and which is believed to be important for proliferation, differentiation and cellular function [13]. Given the role of this protein in iron transportation and binding, its absence in the ULF near non viable embryos could be interpreted as a lack of production of transferrin by the *conceptus*. Transferrin appears to be involved in the formation of blood vessels [13], which then could account for the absence of those structures in the membranes of non viable *conceptuses*.

At all gestational ages studied, proteins identified at spots 47-53 and 63-67, as well as protein at spot 210-213 coincide with reports by Lee et al. [13]. The latter, is important for the establishment of pregnancy according to Lee et al. [13].

Proteins encountered during the follicular and luteal phases of the estrous cycles (Rodríguez and Hernández, unpublished data) were also found in viable embryos at 28 and 35 day-old embryos. At these stages of gestation the protein profile in the ULF showed quantitative differences, since there were grater amounts of protein near viable embryos than in the correspondent sites of non viable ones (FIG. 2 and 3).

The electrophoretic profile became more complex with the advancement of gestation, from MW ~148 proteins at 20 days, up to ~198 at day 35 of gestation. Also, with the advancement of gestation, the complexity of the protein profile in the uterine luminal fluid increases, coincidently, trophoblastic growth becomes evident, spreading from embryonic sites, towards the extremities of the *conceptus* [5, 16]. It is also clear, that glandular growth is going on simultaneously. Godkin et al. [5], also found an increment in protein secretion *in vitro*, concomitant with placental development. As pregnancy progresses, the pattern of protein secretion to the uterine lumen, becomes more uniform.



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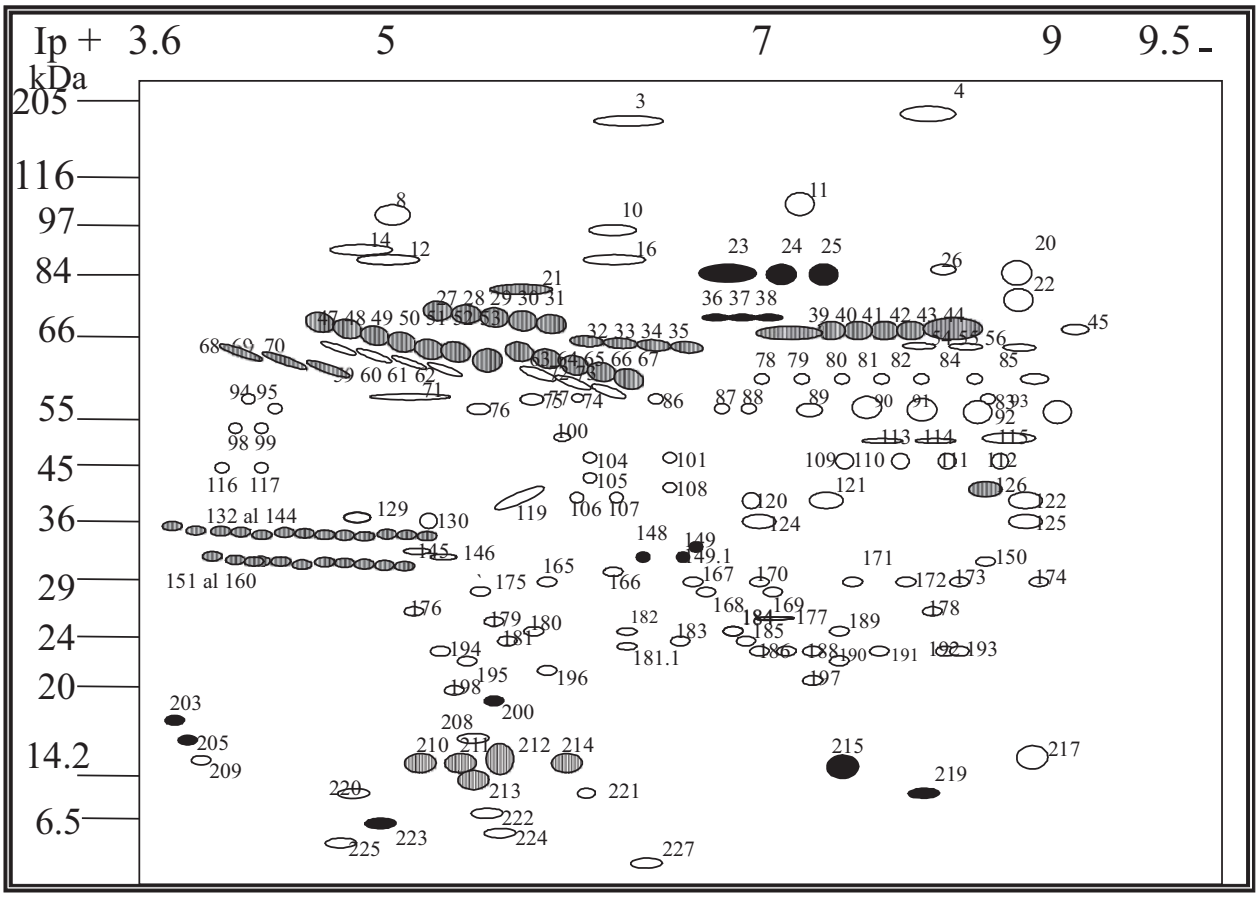

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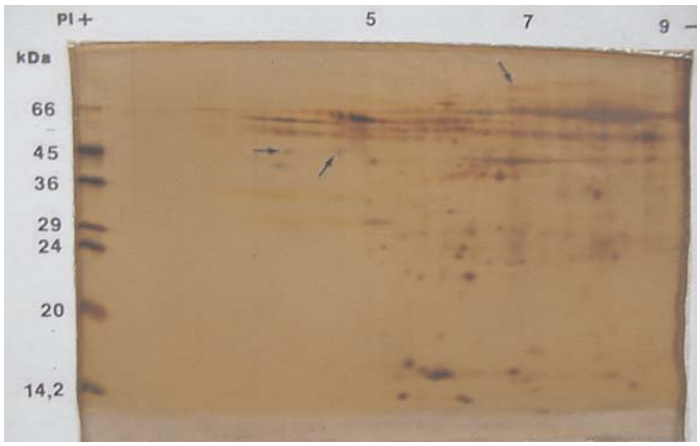
FIGURE 2. 2D-SDS-PAGE. DETECTED PROTEINS IN THE UTERINE LUMINAL FLUID AT 28 DAYS OF GESTATION, (A) CORRESPONDING TO VIABLE EMBRION'FLU AND (B) NON VIABLE EMBRION. QUALITATIVES DIFFERENCES ARE REPRESENTED BY THE ARROWS. (C) SUMMARY OF THE TOTAL NUMBER OF DETECTED PROTEINS IN THE UTERINE LUMINAL FLUID AT 28 DAYS OF GESTATION. PROTEINS PRESENT IN ALL EMBRYOS ARE SHOWN AS ○. QUALITATIVE DIFFERENCES, AS ● (ONLY FOUND IN VIABLE EMBRYOS). QUANTITATIVE DIFFERENCES ARE SHOWN AS ◍ (VIABLE EMBRYOS > NON-VIABLE ONES) AT THE LEFT SIDE APPEAR MOLECULAR WEIGHT MARKERS AND THE SEPARATION CHARGE PRODUCED BY ISOELECTROFOCUSING IS SHOWN AT THE TOP OF THE FIGURE. MOLECULAR MASS MARKERS ARE INDICATED ON THE LEFT.



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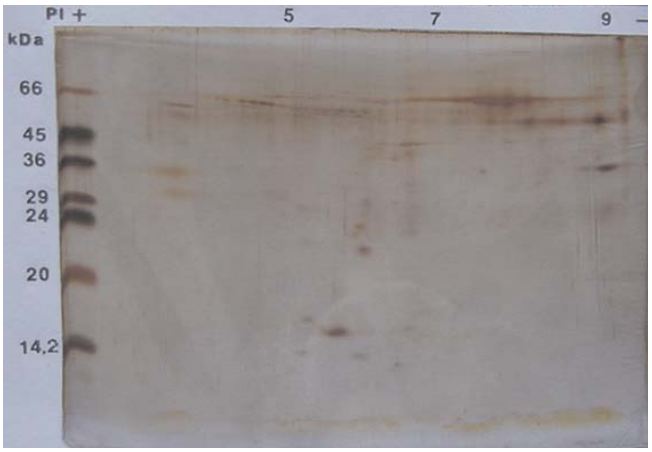

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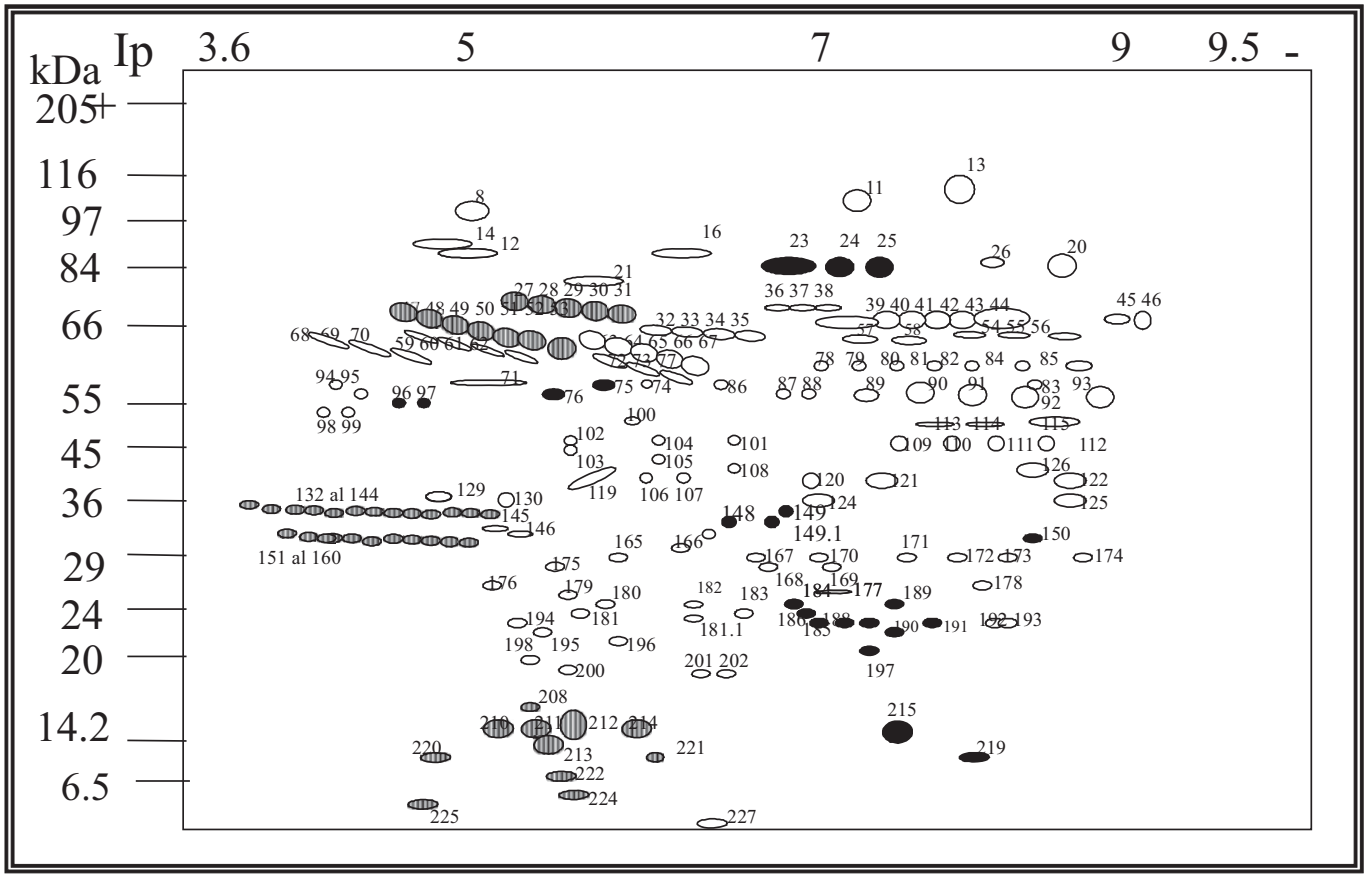

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FIGURE 3. 2D-SDS-PAGE. DETECTED PROTEINS IN THE UTERINE LUMINAL FLUID AT 35 DAYS OF GESTATION, (A) CORRESPONDING TO VIABLE EMBRION'FLU AND (B) NON VIABLE EMBRION. QUALITATIVES DIFFERENCES ARE REPRESENTED BY THE ARROWS. (C) SUMMARY THE TOTAL NUMBER OF DETECTED PROTEINS IN THE UTERINE LUMINAL FLUID AT 35 DAYS OF GESTATION. PROTEINS PRESENT IN ALL EMBRYOS ARE SHOWN AS ○. QUALITATIVE DIFFERENCES ARE SHOWN AS ● (ONLY FOUND IN VIABLE EMBRYOS). QUANTITATIVE DIFFERENCES ARE SHOWN AS ◍ (VIABLE EMBRYOS > NON-VIABLE ONES) AT THE LEFT SIDE APPEAR MOLECULAR WEIGHT MARKERS AND THE SEPARATION CHARGE PRODUCED BY ISOELECTROFOCUSING IS SHOWN AL THE TOP OF THE FIGURE. MOLECULAR MASS MARKERS ARE INDICATED ON THE LEFT.

Quantitative differences among viable and non-viable embryos, at different gestational ages, are shown in FIG. 1, 2, and 3.

Under the conditions of the present trial, at all gestational stages studied, proteins similar to IFN-t, and other proteins which are dependent for their secretion on IFN-t release, like ubiquitin cross-reactive protein (UCRP) [7, 10, 11, 19] were absent, both in viable and non-viable *conceptuses*, which could be explained because interpheron-t secretion ends by the 21st day of gestation [1]. In the present study, IFN-t was not found at day 20. Also, in the present model of induced multiple pregnancy, some unidentified changes could imply modifications of gene expressions.

Proteins with MW and Ip compatible with ovine Placental lactogen (oPL) and pregnancy specific protein B were not detected. This could be explained accepting the proposal that they are presumed to be secreted to the endometrium, rather than to the uterine lumen [2, 20].

CONCLUSIONS

There are some proteins in viable embryos, which are absent in non-viable ones, at 20, 28, and 35 days of gestation. They could represent key molecules to determine the viability of embryos, although some of them have not been identified yet. It is to be expected that with cellular death and/or absence of development in the trophoblast, several secretory products would disappear, which could as well be inductors to the secretion of proteins by the uterus.

It is also possible that embryonic death is the result of lack of some proteins secreted by the *conceptus* or the uterus. In the latter event, this might involve a localized event, to explain present findings, since embryonic decay was not generalized.

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