

## Different influence of ovine estrus synchronization treatments on caruncular early angiogenesis

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**Summary.** The present study compares two protocols for ovine estrus synchronization by assessing the caruncular angiogenic response to the establishment of pregnancy. The analysis consisted of the immunohistochemical evaluation of Vascular Endothelial Growth Factor (VEGF), Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1, CD31) and Von Willebrand Factor (vWF) in ovine caruncular stroma. A flock of thirty-eight adult ewes was divided in two groups and synchronized with either progestagens (Group P) or prostaglandin analogues (Group PG). Immunohistochemistry was performed in uterine samples obtained from pregnant ewes (P, n=15; PG, n=13) on days 15 post coitus (pc), 17pc and 21pc (day 0 =day of estrus). Each factor was assessed by total vascular density (TVD, total positive blood vessels/mm<sup>2</sup>), capillary vascular density (CVD, positive blood capillaries/mm<sup>2</sup>) and arteriolar vascular density (AVD, positive arterioles/mm<sup>2</sup>). Group P demonstrated higher VEGF-CVD (P=0.045) when compared to prostaglandin treated animals. Vascular CD31-expression decreased on days 15pc and 21pc (TVD, P=0.007 and CVD, P=0.014) in both groups. vWF analysis did not show significant differences between groups or days of study. These results demonstrate a different influence of progestagen-based and prostaglandin analogues-based synchronization treatments over VEGF vascular expression during caruncular development taking place in response to pregnancy establishment. In addition, observations pointed out in this study support the involvement of CD31 in the angiogenic stimulus that occurs during early maternal placentation in the ewe.

**Key words:** Ewe, Angiogenesis, Early placentation, Estrus synchronization

### Introduction

Livestock industries conduct management of the reproductive cycle in order to optimize animal productivity and overcome reproductive failure. Different assisted reproductive technologies are being routinely used, including sex hormone treatments (Bazer and Spencer, 2005). In ruminants, estrus is commonly synchronized with a progestagen-based treatment, which imitates the activity of the corpus luteum (CL), or with prostaglandin-analogues, inducing luteolysis (Inskeep, 1973; Gonzalez-Bulnes et al., 2005; Weems et al., 2006).

However, differences between these two treatments have been found in the ewe. In comparison to prostaglandin analogues, progestagens have shown lower quality of preovulatory follicles (Gonzalez-Bulnes et al., 2005), altered functionality of the induced CL (Letelier et al., 2010), lower oviductal and uterine steroid receptors expression during early pregnancy (García-Palencia et al., 2007) and worse viability of early embryos (Gonzalez-Bulnes et al., 2005). On the other hand, prostaglandin-based protocols have demonstrated supranormal levels of plasma progesterone (Letelier et al., 2010) accompanied by variable fertility rates (Barrett et al., 2002), disrupted pattern of the source of ovulatory follicles and disruption of normal luteogenesis (Liu et al., 2006), impaired uterine receptivity to the embryo (Ruiz-González et al., 2012) and decreased reproductive performance (Olivera-Muzante et al., 2011). Therefore, no definitive conclusion about their impact or suitability for ovine reproductive physiology has yet been achieved.

In mammals, maternal tissues begin remodelling after mating to become a suitable environment for

conceptus implantation (Bowen and Burghardt, 2000; Spencer et al., 2004; Bazer et al., 2009). In the sheep, implantation begins on days 15-16 (Guillomot, 1995), involving maternal caruncles and foetal cotyledons as attachment sites, forming the placentomes, where the maternal-foetal metabolic exchange will take place (Reynolds et al., 2010). Thus, placentation will include extensive angiogenesis in both maternal and foetal placental tissues to ensure the increasing metabolic needs of the developing foetus (Reynolds et al., 2005b; Osol and Mandala, 2009; Grazul-Bilska et al., 2011).

Previous studies have described increased microvascular density in caruncular tissues of the gravid horn, as well as subepithelial microvascular plexuses development in both caruncular and intercaruncular areas of the endometrium (Reynolds and Redmer, 1992). This would allow uterine blood flow to increase from day 11 to 30 in pregnant ewes (Reynolds et al., 1984). Consequently, placental efficiency associates with its vascular density (Reynolds and Redmer, 1995, 2001) and blood flow (Reynolds and Redmer, 1992; Reynolds et al., 2006; Kaczmarek et al., 2009; Grazul-Bilska et al., 2010), since it is known that inadequate placental growth and vascular development is related to impaired implantation (Sherer and Abulafia, 2001), embryo mortality (Reynolds and Redmer, 2001), altered foetal development (Reynolds et al., 2005a; Barry and Anthony, 2008; Ma et al., 2010) pregnancy failure (Zygmunt et al., 2003), and compromised postnatal survival and growth (Millaway et al., 1989; Hafez et al., 2010).

Angiogenesis comprises development of new blood vessels from a pre-existing vascular bed and it depends on numerous elements, including angiogenic factors and cell adhesion molecules (Distler et al., 2003; Ramjaun and Hodivala-Dilke, 2009). Vascular Endothelial Growth Factor (VEGF) is one of the most important angiogenic factors due to its ability to increase permeability of blood vessels, stimulate proliferation/migration of endothelial cells and maintenance of the vascular bed (Ferrara and Davis-Smyth, 1997; Mehta and Malik, 2006; Kaczmarek et al., 2008a; Manisha et al., 2010; Sagsöz and Saruhan, 2011). Involvement of VEGF and its receptors (VEGFR-1 and VEGFR-2) in early pregnancy and placentation has been described in a variety of species, including humans (Sugino et al., 2002; Huppertz and Peeters, 2005), mice (Rabhani and Rogers, 2001; Rockwell et al., 2002; Douglas et al., 2009), rhesus monkey (Ghosh et al., 2000), marmoset (Rowe et al., 2003, 2004), rabbit (Das et al., 1997), pig (Charnock-Jones et al., 2001; Vonnahme et al., 2001; Wollenhaupt et al., 2004; Kaczmarek et al., 2008b), cow (Pfarrer et al., 2006), and sheep (Bogic et al., 2001; Reynolds et al., 2005b, 2010; Grazul-Bilska et al., 2010, 2011).

In addition, Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1, also named as CD31) is known to collaborate in aggregation and migration of endothelial cells, vessel stabilization and vascular

maintenance (Ferrer et al., 1995; DeLisser et al., 1997; Cao et al., 2002; Distler et al., 2003). Rowe et al. (2004) assessed uterine microvascular development in the marmoset using CD31, which had been used to study angiogenesis in tumours (Ferrer et al., 1995), since its expression by early endothelial cells allows researchers to detect newly formed vascular beds (Horak et al., 1992; Weidner, 1995; Lyall et al., 2001). Assessment of von Willebrand Factor (vWF) has been also utilized to identify microvessels in tissue sections (Reynolds and Redmer, 1992; Weidner, 1995), although alone it would not be suitable to study neoangiogenesis since it does not mark small immature microvessels or single endothelial cells (Giatromanolaki et al., 1997). Moreover, augmentation of vWF expression has been associated with vascular damage, maturation and regression of vessels (Woolf et al., 1987, Bowyer et al., 1989; Modlich et al., 1996; Augustin, 1998).

Since many factors and adhesion molecules involved in angiogenesis are known to be influenced by reproductive hormones (Shweiki et al., 1993; Rahman et al., 2004; Hervé et al., 2006; Reisinger et al., 2007; Chang and Zhang, 2008), the impact of assisted hormonal treatments over endometrial angiogenesis has been the purpose of many studies in humans. Contraceptive treatments seemed to diminish VEGF endometrial glandular immunorexpression (Macpherson et al., 1999). Furthermore, microvascular density was reduced in women with hormonal replacement treatment when compared to controls (Hickey et al., 2003). In addition, Jondet et al. (2006) demonstrated that progestin treatments decreased microvascular vessel number in a dose dependent manner, in accordance with the results reported by Okada et al. (2011), where it was demonstrated that progestins inhibited estradiol-mediated VEGF expression *in vitro*.

In sheep, limited data has been published regarding the impact of estrus synchronization treatments on the vascular expansion of the pregnant endometrium, and reports available are focused on CL vascular development (Vonnahme et al., 2006; Letelier et al., 2011). To our knowledge, no previous publications have compared the influence of progestagen-based and prostaglandin-based synchronization treatments over endometrial angiogenesis during pregnancy establishment, which is the period comprising most of the embryonic loss in ruminants (Thatcher et al., 1994; Roberts et al., 1996; Reynolds and Redmer, 2001; Spencer et al., 2008).

Therefore, we hypothesize that estrus synchronization treatments could influence early placental angiogenesis. Disparity in caruncular vascular development could be used to demonstrate this effect. VEGF and CD31 are commonly utilized as indicators of angiogenesis and neovascularization. In addition, vWF has been widely utilized as a vascular marker when studying angiogenesis. Thus, the purpose of the present work was to study vascular immunohistochemical expression of VEGF, CD31 and vWF in ovine

## Treatment effect on early angiogenesis

caruncular tissues (maternal placenta) during early pregnancy, comparing between ewes whose estrus was synchronized with either prostaglandin-based or progestagen-based treatment.

### Materials and methods

#### Animal experimental design and collection of samples

For this study 38 adult, normally cycling, multiparous Manchega ewes were used. Females were maintained at the experimental farm of the Animal Reproduction Department at the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA, Madrid, Spain; latitude 40°25'N). These facilities meet the requirements for Scientific Procedure Establishments and Animal Protection determined by the European Union. The current experiment was performed after approval from the Scientific Ethic Committee during the natural breeding season (October-March) described for this breed at this latitude.

In group P, ewes were treated with progestagens impregnated in an intravaginal pessary (40 mg fluorogestone acetate, FGA, Chronogest<sup>®</sup>, Intervet International, Boxmeer, The Netherlands), for 14 days. In group PG, ovarian cyclic activity was synchronized by inducing luteolysis with cloprostenol, a prostaglandin F<sub>2</sub>α analogue (Estrumate, Mallinckrodt Vet GmbH, Friesoythe, Germany), in two intramuscular (i.m.) doses of 100 µg given 10 days apart. Estrous behavior was determined every 6 h, from 18 to 42 h after the second cloprostenol injection or the sponge withdrawal, by using adult rams at a rate of one ewe/one male. When a ewe was detected in estrus, mating was allowed to be repeated between 6 and 12 hours later in order to ensure fertilization.

The number of sheep responding to the synchronization treatment and showing signs of estrus was similar in both groups [74.4% in group P (n=16) and 82% in group PG (n=14)]. Animals from either group that did not show signs of estrus by the time of 42h were not included in the study.

The day of first detection of estrus signs and mating

was considered Day 0 for experimental purposes. Afterwards, the presence of at least one corpus luteum in each sheep was assessed daily until day 21pc using 7.5 MHz transrectal ultrasonography (Aloka SSD-500, Ecotron, Madrid, Spain). Efficiency of mating in this flock was around 92% of the sheep that responded to either synchronization treatment.

The reproductive tract of pregnant animals was immediately removed after euthanasia with an overdose of T 61<sup>®</sup> (Hoechst-Roussel Vet. A.I.E.) on days 15pc (P n=5, PG n=4), 17pc (P n=5, PG n=5) and 21pc (P n=5, PG n=4). A section of each uterine horn was obtained (from the same location in all animals), fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, sectioned at 4 µm, stained with haematoxylin–eosin and evaluated microscopically.

#### Immunohistochemistry

Immunohistochemistry was performed on 4 µm de-waxed sections of the uterus by the streptavidin–biotin peroxidase complex method using antibodies against VEGF, CD31 and vWF to detect their expression. For the assessment of these three proteins, slides were deparaffinized, rehydrated through a series of graded volumes of ethanol and washed in distilled water (5 minutes). The protocols followed for each analysis have been summarized in Table 1.

#### Vascular Endothelial Growth Factor (VEGF)

Deparaffinized tissue sections for VEGF were pretreated with 10mM Citrate buffer (pH 6.0) in a pressure cooker for antigen retrieval. Slides were cooled for 30 min and washed twice in distilled water. Non-specific endogenous peroxidase activity was blocked by treatment with 33% hydrogen peroxide in methanol at 1.5% for 15 min at room temperature (RT). Tissue sections were washed twice in distilled water and then twice in TBS Tween (0.05%). Slides were incubated in a humidified chamber at 4°C overnight with anti-VEGF mouse monoclonal antibody (VEGF (C-1) sc-7269, Santa Cruz Biotechnology, Inc.) at 1:25 dilution in TBS.

**Table 1.** Summary of the protocols followed for vascular VEGF, CD31 and vWF immunolocalization in tissue sections of ovine pregnant uterus.

	Buffer	Antigen Retrieval	Peroxidase Block	Normal Serum	Secondary Antibody	Streptavidin
VEGF	10 mM Citrate Buffer pH 6.0	Pressure Cooker	1.5% H <sub>2</sub> O <sub>2</sub> in Methanol 15 min, RT	None	1:400, 30 min, RT <sup>3</sup>	1:400, 30 min, RT <sup>5</sup>
CD31	1 mM EDTA pH 8.0	Microwave 750W 3 x 5 min	Peroxidase Block <sup>1</sup> 5 min, RT	None	Post primary block <sup>1</sup> , 30 min, RT	Novolink™ Polymer <sup>1</sup> 30 min, RT
vWF	10 mM Citrate Buffer pH 6.0	Trypsin 37°C 10 min+ Microwave 750W 5min	1.5% H <sub>2</sub> O <sub>2</sub> in Methanol 15 min, RT	1:20, 30 min, RT <sup>2</sup>	1:200, 30 min, RT <sup>4</sup>	1:400, 45 min, RT <sup>5</sup>

<sup>1</sup>Novolink Polymer Detection System, N°RE7140-K, Leica Microsystems, United Kingdom. <sup>2</sup>Swine normal serum Code N°X0901, Dako A/S, Denmark.

<sup>3</sup>Horse anti-mouse Immunoglobulins/biotinylated Vector Laboratories Ltd., Burlingame, CA, USA. <sup>4</sup>Polyclonal swine anti-rabbit Immunoglobulins/biotinylated Code N°E0353, Dako A/S, Denmark. <sup>5</sup>Streptavidin conjugate with peroxidase (Zymed, Cat. N°43-4323, Madrid, Spain)

### Platelet Endothelial Cell Adhesion Molecule-1 (CD31/PECAM-1)

Deparaffinated samples were pretreated in 1mM EDTA buffer (pH 8.0) in a microwave oven at 750 W, three cycles (5 min each), for antigen retrieval. Slides were cooled during 30 min then washed twice in distilled water. The protocol continues as recommended in the Instructions for Use of the Novolink working kit (NovoLink Polymer Detection System, 250 tests, N°RE7140-K, Leica Microsystems, United Kingdom). Primary antibody (CD31 Rabbit Polyclonal antibody, Ca N°250590, Abbiotec, LLC, San Diego, CA.) was diluted 1:300 in diluent (25% Novolink Protein Block Reactive, 75% TBS) for 1 hour at RT. Afterwards, sections were incubated with Novolink Post-Primary Block for 30 minutes (RT), washed twice in TBS Tween, and incubated with Novolink Polymer for 30 minutes (RT). They were washed again twice in TBS Tween, rocking gently as specified in the working kit instructions.

### von Willebrand Factor (vWF)

Deparaffinized samples were pretreated with Trypsin in TBS at 37°C for 10 min. Slides were washed twice in distilled water, and then treated in a microwave oven at 750W (1 cycle, 5 minutes) with 10mM sodium citrate buffer (pH 6.0). Slides were cooled during 30 min and washed twice in distilled water. Non-specific endogenous peroxidase activity was blocked by treatment with 33% hydrogen peroxide in methanol at 1.5% for 15 min at RT. Tissue sections were washed twice in distilled water and then twice in TBS Tween (0.05%). A swine normal serum (Code No.X0901, Dako A/S, Denmark) was used at 1:20 dilution in TBS for 30 min at RT. Slides were then incubated overnight at 4°C with rabbit polyclonal antibody raised against human von Willebrand Factor (Code No A0082, Lot 105, DAKO A/S, Denmark) at 1:2000 diluted in TBS.

### Development, counterstaining and mounting

For the three proteins analyzed, tissue sections were washed twice in TBS Tween (5 minutes each time) after the primary antibody. Following incubation with the correspondent secondary antibody (as detailed in Table 1), slides were washed twice in TBS Tween and incubated with the specified streptavidin conjugate with peroxidase (as shown in Table 1).

Then, they were washed again in TBS Tween and developed with 3-3' diaminobenzidine tetrachloride (DAB) (DAB kit, K4100, Vector Laboratories Ltd., Burlingame, CA, USA). After a 10 min wash in distilled water, tissue sections for VEGF, CD31 and vWF were counterstained with haematoxylin, dehydrated in alcohol, cleared and mounted. For the negative control sections, equivalent concentration of non-immune mouse IgG or rabbit IgG antibody (Dakocytomation, Cat. N°Z0259; BD Diagnostics, Sparks, MD) was used

instead of the primary antibody.

### Image analysis

A subjective image analysis to estimate the number of vessels positive for VEGF, CD31 and vWF was performed by two observers who were not aware of which group of animals was being analyzed. Positive caruncular blood vessels were studied on days 15pc, 17pc and 21pc, including: Total Vascular Density (TVD, total positive blood vessels/mm<sup>2</sup>), Capillary Vascular Density (CVD, positive blood capillaries/mm<sup>2</sup>), Arteriolar Vascular Density (AVD, positive arterioles/mm<sup>2</sup>). Blood vessels showing staining were marked as positive using Image J Software. Six fields in each sample were examined at 400x magnification. The area of caruncular tissue included in each field was 0.019671 mm<sup>2</sup>. A mean number per unit (mm<sup>2</sup>) was assigned to each animal after assessing both uterine horns. Images were obtained using an Olympus camera connected to an Olympus DP50 microscope and processed in a computer using both Viewfinder Lite<sup>®</sup> and StudioLite<sup>®</sup> programmes (Better Light Inc, San Carlos, CA).

### Statistical analysis

Results obtained were analyzed at the Centro de Cálculo of the Universidad Complutense de Madrid, using SPSS Statistics 19 Software for Windows (SPSS-Ibérica Inc., Spain). Two way-analysis of variance (ANOVA) was used to compare mean density (total density, capillary density and arteriolar density) of each factor (CD31, VEGF and vWF) regarding the different variables considered (density by treatment and density by day post-coitus). Pearson correlation was determined between the different parameters of each factor. In addition, Duncan *post hoc* analysis was performed to study in depth the significant differences found between days of pregnancy. All results are expressed as mean ± standard error of mean (s.e.m.) and were considered statistically significant when P<0.05.

## Results

### VEGF

Blood vessels from the caruncular area positive to VEGF showed brownish cytoplasmic staining in endothelial cells (Fig. 1). Smooth muscle cells also stained positive in larger blood vessels. Results regarding VEGF vascular densities in the caruncular stroma have been compiled in Figure 2.

No significant differences were obtained between treatments or days of pregnancy regarding total VEGF-positive blood vascular density, although the pattern of expression observed was different in both groups. TVD decreased continuously from day 15pc to 21pc in the PG group. In the P group, VEGF expression decreased from

### Treatment effect on early angiogenesis

day 15pc to 17pc, but increased afterwards until day 21pc.

Differences between treatments were obtained in the capillary vascular density ( $P=0.045$ ), with CVD in group P being significantly higher than in group PG. In the group treated with progestagens, the number of capillaries positive to VEGF increased from day 15 to 21pc. However, in the group treated with prostaglandin analogues, CVD decreased from day 15pc to day 21pc. Differences in VEGF capillary density between days of pregnancy were not significant.

No differences were found between treatments or between days of pregnancy regarding arteriolar vascular density ( $P>0.05$ ). However, the staining pattern observed as pregnancy advanced was different between both groups. In the PG group, positive vessels decreased from day 15pc to day 21pc. On the other hand, the progestagen-treated group demonstrated decreasing expression from day 15pc to 17pc, only to increase afterwards from day 17pc to day 21pc.

In the PG group, VEGF-Total vascular density correlated mildly with the CVD ( $r^2= +0.566$ ,  $P=0.044$ ) and highly with the AVD ( $r^2= +0.944$ ,  $P=0.000$ ). In the group treated with progestagens, total density followed the same pattern, although correlations were lower than group PG for the capillary density ( $r^2= +0.526$ ,  $P=0.044$ )

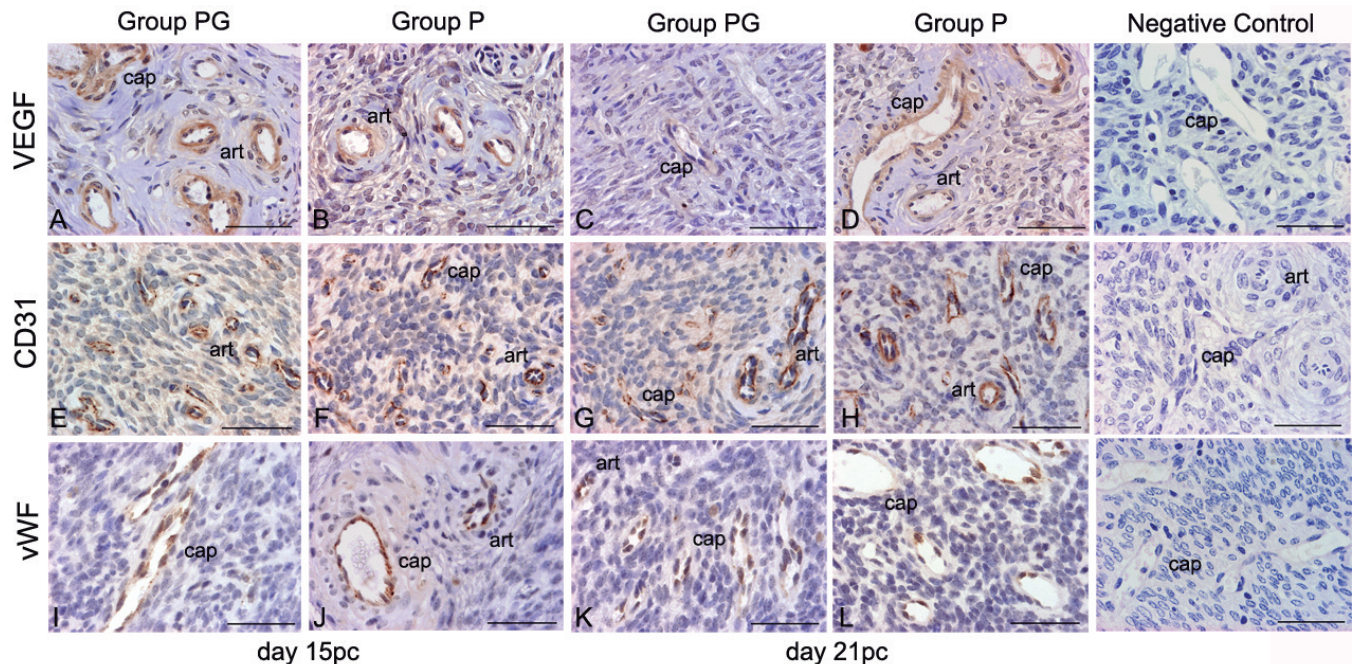
and the arteriolar density ( $r^2= +0.888$ ,  $P=0.000$ ).

#### CD31

Caruncular CD31-positive blood vessels showed a brownish cytoplasmic staining in the endothelial cells (Fig. 1). Results regarding CD31-total, capillary and arteriolar vascular densities have been summarized in Figure 2.

When comparing days of pregnancy, a significant decrease of CD31-TVD was observed ( $P=0.007$ ). Duncan *post hoc* analysis demonstrated that total vascular density on day 15pc was similar to day 17pc, although both days differed significantly from day 21pc. Although no significant differences were demonstrated between treatments, the decreasing pattern of CD31-TVD differed in both groups. Positive blood vessels in the prostaglandin-treated group increased slightly from day 15pc to 17pc, and then decreased until day 21pc. However, CD31-TVD in group P decreased from day 15pc to 21pc.

CD31-capillary vascular density differed as pregnancy advanced ( $P=0.014$ ). *Post hoc* analysis revealed that expression on day 15pc differed significantly from day 21pc. No significant differences were demonstrated between treatments. However, on



**Fig. 1.** Representative images showing VEGF, CD31 and vWF vascular immunostaining in the ovine caruncle. **A-D.** Apparent immunoreactive VEGF labeling on day 15pc in group PG (**A**) and in group P (**B**). Capillary staining on day 21pc is decreased in group PG (**C**) when compared to group P (**D**). **E-H.** Higher density of CD31-positive vessels on day 15pc (**E, F**) in comparison to day 21pc (**G, H**) in both groups. **I-L.** Immunoreactive vWF increases from day 15pc (**I-J**) to 21pc (**K-L**) in both groups of treatment. Streptavidin-biotin-peroxidase complex method; cap, capillary; art, arteriole. Bar: 40  $\mu\text{m}$ .

Treatment effect on early angiogenesis

day 15pc, CD31-CVD was higher in the progestagen group in comparison to group PG. On day 17pc, CD31-capillary density decreased in the progestagen-treated group, although it remained constant in group PG. Finally, on day 21pc, both groups showed decreased CVD in comparison to day 15pc.

Arteriolar vascular density did not show significant differences between groups of treatment or between days of pregnancy ( $P>0.05$ )

In the PG group, CD31-total vascular density was strongly correlated with CVD ( $r^2= +0.951$ ,  $P=0.000$ ) and AVD ( $r^2= +0.795$ ,  $P=0.001$ ). In addition, this group also demonstrated high correlation between CVD and AVD ( $r^2= +0.807$ ,  $P=0.001$ ). However, in the progestagen treated group, total vascular density correlation with capillary density was slightly higher ( $r^2= +0.986$ ,  $P=0.000$ ), but it did not correlate with the arteriolar density. Moreover, the P group demonstrated a negative correlation between CD31-CVD and VEGF-TVD ( $r^2=$

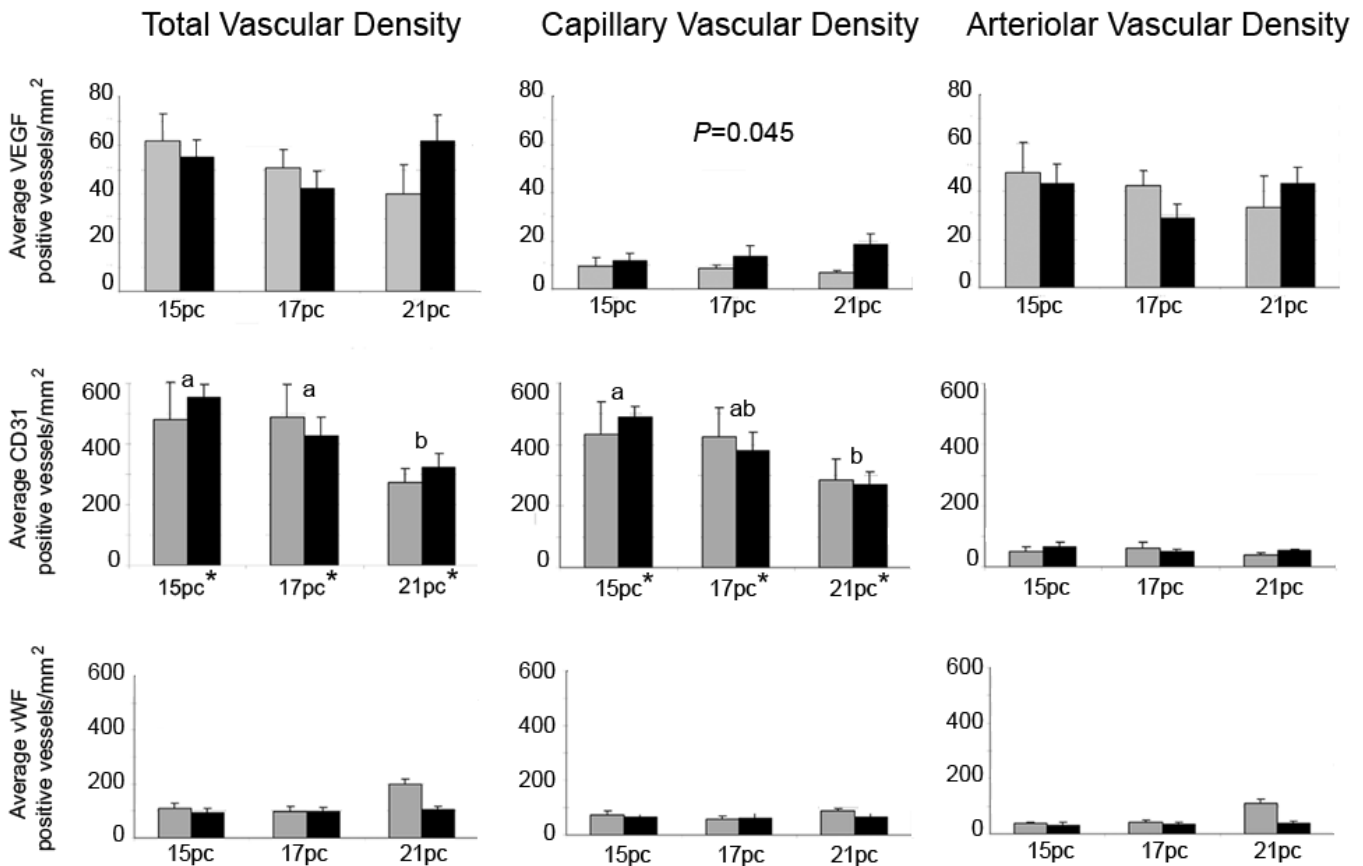
$-0.535$ ,  $P=0.04$ ) that was not observed in the group treated with prostaglandin analogues.

vWF

vWF-positive caruncular blood vessels showed brownish staining in the endothelial cells (Fig. 1). Results regarding vWF vascular densities have been compiled in Fig. 2.

No significant differences were obtained when assessing vWF immunorexpression regarding treatment or days of pregnancy in any of the parameters studied. However, although staining of immunoreactive vWF remained constant on days 15pc and 17pc in both groups, expression on day 21pc was higher in the PG group for every parameter studied.

In the prostaglandin-treated group, vWF-total vascular density was strongly correlated with CVD ( $r^2= +0.873$ ,  $P=0.000$ ) and with AVD ( $r^2= +0.826$ ,  $P=0.001$ ).



**Fig. 2.** Caruncular VEGF, CD31 and vWF vascular immunoreactivity represented as total vascular density (TVD), capillary vascular density (CVD) and arteriolar vascular density (AVD) on days 15pc, 17pc and 21pc in ewes treated with prostaglandin analogues (grey bars) and progestagens (solid black bars). Results are expressed by mean  $\pm$  s.e.m., and were considered statistically significant when  $P<0.05$ . Significant differences between treatments regarding vascular immunostaining are marked with (\*). Different superscript (<sup>a,b</sup>) specifies days with similar significance.

## Treatment effect on early angiogenesis

In the P group, total vascular density followed the same pattern as in the PG group, being strongly correlated with CVD ( $r^2 = +0.844$ ,  $P = 0.000$ ). However, total density was less positively correlated with arteriolar density ( $r^2 = +0.600$ ,  $P = 0.018$ ) than in group PG.

### Discussion

Results obtained in the present work demonstrate that VEGF vascular expression is different depending on the protocol used for ovine estrus synchronization. In addition, it has been revealed that CD31-density changes as pregnancy is established, supporting its implication in the caruncular angiogenic development that takes place in this early phase of gestation.

In our study, progestagen-treated ewes demonstrated a higher number of capillary-type vessels positive to VEGF than the animals synchronized with prostaglandin analogues. It has been mentioned that VEGF regulates vascular development and permeability during early pregnancy, as an increased VEGF-system expression in ovine maternal and foetal placentas has been observed (Grazul-Bilska et al., 2010, 2011), as well as its involvement in the peri-implantational stage and pregnancy maintenance of many species (Wollenhaupt et al., 2004; Huppertz and Peeters, 2005; Pfarrer et al., 2006). Since it is known that ongoing abnormalities in the VEGF-system induce changes in placental vascular development (Reynolds et al., 2005b; Pfarrer et al., 2006; Douglas et al., 2009) that could lead to compromised implantation, abortion, alterations of foetal development or pregnancy loss (Sherer and Abulafia, 2001; Zygmunt et al., 2003; Reynolds et al., 2005a, 2010), the reduced VEGF-density observed in the PG group during days 15-21pc would indicate an impaired vascular development and remodelling of the caruncular tissue in those ewes synchronized with prostaglandin analogue protocols.

The VEGF-mediated vascular remodelling and size increase that takes place during early placentation (Reynolds and Redmer, 1992; Ghosh et al., 2000; Grazul-Bilska et al., 2010; Reynolds et al., 2010) occurs after the creation of a newly formed vascular bed (Reynolds and Redmer, 1992; Rowe et al., 2004; Grazul-Bilska et al., 2010), which would be highlighted by CD31 vascular staining. In our study, the significant decrease that has been observed in CD31-densities from day 15 to 21pc would indicate an initial rise in the number of endothelial cells (Rowe et al., 2004; Grazul-Bilska et al., 2010) and a preliminary high vascular proliferative activity (Demir et al., 2007), which would be lost progressively as vessels remodel and evolve due to an increasing VEGF influence (Yancopoulos et al., 2000; Bogic et al., 2001; Hofer et al., 2006; Mehta and Malik, 2006).

Although not statistically significant, the group synchronized with prostaglandin analogues showed higher, and more sustained, CD31 immunoreactivity between days 15-17pc than the ewes from group P. We

expected that this higher density of newly formed CD31-positive vessels would be replaced by enhanced VEGF-density, which would illustrate a stronger remodelling stimulus over these vessels. This phenomenon was apparent in the progestagens group. Nevertheless, the parallel fall of both CD31 and VEGF densities between days 15-21pc observed in group PG would indicate an impaired relay between neovessel formation and their remodelling or maturation under VEGF influence.

Since steroid hormones are known to exert an effect over vascular adaptations during pregnancy establishment (Shweiki et al., 1993; Hyder and Stancel, 1999; Sugino et al., 2002; Wollenhaupt et al., 2004; Reynolds et al., 2005b; Johnson et al., 2006; Kaczmarek et al., 2008a), the differences pointed out here between both treatments could be related to variations in the hormonal profile that is being displayed during early pregnancy, which changes depending on the protocol utilized for estrus synchronization. The higher levels of estradiol (García-Palencia et al., 2007) and plasmatic progesterone (Letelier et al., 2010; Ruiz-González et al., 2012) showed early after mating by prostaglandin-synchronized ewes would predispose these animals to develop a maternal-fetal asynchrony (Lawson et al., 1983; Satterfield et al., 2006; Clemente et al., 2009) that could compromise pregnancy establishment and its maintenance (Spencer and Bazer, 2004; Silva et al., 2011; Ruiz-González et al., 2012). An ongoing asynchrony between the embryo and the maternal environment in group PG could be altering the materno-foetal signalling required to stimulate angiogenesis between days 15pc and 21pc, a period considered critical in sheep since it comprises maternal recognition of pregnancy, embryo implantation and initiation of placental development (Spencer et al., 1995, 2004; Roberts, 2007).

In addition, uterine stromal cells have been implicated in the processes of gestational neovascularization (Sugino et al., 2002; Rowe et al., 2003; Nishimura et al., 2004; Wei et al., 2004; Laws et al., 2008; Nair et al., 2011) since they continue to express PR after its loss in the superficial epithelium (Spencer et al., 2004; Satterfield et al., 2008, 2009; Bazer et al., 2010). The apparent loss of the angiogenic stimuli in the PG group, illustrated by decreasing VEGF-densities, could be related to the lower stromal PR-immunoreactive protein levels demonstrated by ewes synchronized with prostaglandins in comparison to those treated with progestagens (unpublished results).

Although not significantly, vWF expression increased in group PG from day 15 to day 21pc. As observed in buffalo atretic follicles, vWF increases consequently to blood vessel maturation and aging (Ferani et al., 2005), as well as in situations related to endothelial damage (Reynolds and Redmer, 1992; Hickey and Fraser, 2000; Shimizu et al., 2003). Since it has been described that contraceptive treatments influence vWF expression (Crowell et al., 1971; Harrison and McKee, 1984), its increasing

immunostaining observed in group PG, in combination with the loss of CD31 and VEGF, could be indicating a predominance of maturation stimuli over those of proliferation and remodelling in the group synchronized with prostaglandin analogues. As has been mentioned, abnormal vascular development and blood flow would compromise pregnancy (Reynolds et al., 2006, 2010; Hafez et al., 2010). This ongoing condition in the PG group would contribute to explain the variable pregnancy rates and the lower birth rates described when synchronizing ewes with prostaglandin analogue-based protocols (Barrett et al., 2002; Olivera-Muzante et al., 2011).

In conclusion, the results obtained in this study demonstrate that some aspects of the angiogenic stimulus taking place in caruncular tissues during ovine early placentation are indeed influenced by the treatment used for estrus synchronization. Observations pointed out here indicate that VEGF caruncular density during pregnancy establishment is reduced in ewes synchronized with prostaglandin analogues in comparison to progestagens. In addition, we demonstrate an active involvement of CD31 in the vascular development occurring in the ovine caruncle during early placentation. Unparalleled caruncular vascular development during this early phase of gestation could contribute to explain previous differences obtained when progestagen-based estrus synchronization protocols were compared to prostaglandin analogues in the ewe.

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