Undernutrition and laterality of the corpus luteum affects gene expression in oviduct and uterus of pregnant ewes

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Abstract

The effect of undernutrition on gene expression of progesterone and oestrogen receptors (*PGR* and *ESR1*), and insulin-like growth factors 1 and 2 (*IGF1* and *IGF2*) in the uterus and oviducts of ewes on day 5 after oestrus was investigated. The effect of the side of the uterus/oviduct regarding the ovary bearing a corpus luteum (CL) (ipsi *vs*. contralateral) was also analyzed. Fourteen oestrous synchronized ewes were fed either 1.5 (C, n = 7) or 0.5 (L, n = 7) times their maintenance requirements from the onset of the hormonal treatment (day –14), till slaughter on day 5 post-oestrus. Oviducts and samples of uterus were collected and their gene expression studied by real time RT-PCR. Undernourished ewes had greater *PGR* expression in the oviduct than control ewes, but lower expression of *IGF1* in uterus and of *IGF2* in oviducts. The ipsilateral oviduct presented lower expression of *PGR, ESR1* and *IGF*2 mRNA than the contralateral one, but this did not occur in the uterus. In conclusion, there is an effect of undernutrition on gene expression that is transcript and organ dependent (uterus/oviduct). This work reports for the first time that growth factors and sex steroid receptor expression on day 5 after oestrus vary depending on the side of the CL-bearing ovary and the region of the reproductive tract.

Additional key words: nutrition; reproductive tract; steroid receptors; growth factors.

Introduction

Progesterone (P_4) is the main steroid hormone synthesized by the corpus luteum (CL), and plays a major role in preparing the uterus for pregnancy by promoting and controlling the secretion of uterine proteins and growth factors, the growth rate of embryos and the embryonic secretion of interferon-tau (Spencer & Bazer, 1995; Weems *et al.*, 2011). If the secretion of interferon-tau by the embryo is not delivered appropriately, endometrial prostaglandin F2α is released,

inducing luteolysis, which ends in embryo death (Bonnin *et al.*, 1999).

Undernutrition decreases oocyte quality, embryonic development and pregnancy rates (for a review, see Abecia *et al.*, 2006), and it is well known that feeding below the requirements for maintenance increases peripheral P_4 concentrations (Abecia *et al.*, 1996; Rhind *et al.*, 1985). However, Lozano *et al.* (1998) demonstrated a reduction of P_4 concentration in the endometrium consistent with a lower endometrial abundance of progesterone receptor (PGR) (Sosa *et al.*, 2004) and

the impairment of early embryonic development (Abecia *et al.*, 1995). A differential sensitivity of the reproductive tract to steroids may result in a distinct environment for the embryo, through different growth factors concentrations for instance, as these are mainly regulated by steroids (Sahlin, 1998; Wathes *et al.*, 1998). Both insulin-like growth factors (IGF) I and II are crucial for embryo development (Wathes *et al.*, 1998), and are implicated as regulators of pre-implantation and placental development (Kaye *et al.*, 1992). Despite their relevant role, the effects of undernutrition on the expression of these growth factors along the reproductive tract during the early luteal phase are unknown.

Ovarian venous P_4 pass into the ovarian artery, via a countercurrent mechanism (Einer-Jensen & McCracken, 1981), and a branch of this artery supplies the oviduct/uterus with blood (Del Campo & Ginther, 1973). This results in a gradient of P_4 concentration along the reproductive tract; the closer to the CL, the greater P_4 concentration is (Weems *et al.*, 1989). Abecia *et al.* (1997) found a 30-fold higher concentration of P_4 in the ipsilateral ovarian vein than in the contralateral one. The hormonal environment within or between uterine cornua may influence early embryonic development (Weems *et al.*, 1989). Indeed, embryos transferred into the uterine horn adjacent to the CL result in higher pregnancy rates and proper embryonic development, than when transferred into the contralateral one (Del Campo *et al.*, 1979; Moreno *et al.*, 2003). If a differential plasma steroid supply takes place in the ovine reproductive tract, a distinct gene expression may be expected. There is scarce information about the influence of the side of CL upon gene expression in the uterus and oviduct, and/or differences among gene expression in these two tissues.

This work studied the effects of undernutrition on *PGR*, oestrogen receptor 1 (*ESR1*), *IGF1* and *IGF2* mRNAs, in the reproductive tract (oviduct and uterus) of sheep on day 5 post-oestrus. Moreover, we aimed to analyze the importance of the local effect of CL on gene expression of both oviducts and uterine horns, and elucidate the changes that occur between organs.

Material and methods

Animals and treatments

This study was carried out at the experimental farm of the University of Zaragoza, Spain (latitude 41° N and longitude 0° W) under approval of the Ethics Committee for Animal Experiments of the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

During the breeding season (December-March), 14 Rasa Aragonesa ewes with a mean ($\pm$ SEM) body weight (BW) of 61.9 ± 1.6 kg and an initial body condition score (BCS) above 2.75 (scale from 0 to 5; Russel *et al.*, 1969) received a diet that covered the daily energy and protein requirements (AFRC, 1993), to maintain a constant BCS during 35 days. The diet consisted of 0.45 kg of pellets and 0.55 kg of barley straw per ewe per day providing 8.37 MJ of metabolizable energy kg^{-1} of dry matter (ME kg^{-1} of DM) and 9% crude protein (CP). The pelleted diet consisted of barley (85%) and soybean (15%). After this period, ewes were randomly assigned into two groups and were fed to provide either 1.5 (Control, C, n=7) or 0.5 (Low, L, n=7) times the daily requirements for maintenance. For group C, the daily allocation comprised 0.60 kg of pellets and 0.90 kg of barley straw per ewe per day and for group L, the daily allocation was 0.20 kg of pellets and 0.30 kg of barley straw per ewe per day. Both diets provided 11% of CP and 8.80 MJ ME kg^{-1} (CP: 0.17 *vs.* 0.06 kg day^{-1} for C and L groups respectively, and ME: 13.20 *vs.* 4.40 MJ day^{-1} for C and L group respectively). Body weight and BCS were measured at the beginning of the experiment (day –15) and at slaughter (day 5).

At the beginning of the differential diets, estrus was synchronized by intravaginal progestagen pessaries (fluorogestone acetate, Sincropart® Ceva Animal Health, Barcelona, Spain) for 14 days. At pessary withdrawal, ewes were injected i.m. with 300 IU of equine chorionic gonadotrophin (Sincropart® Ceva Animal Health, Barcelona, Spain) to induce ovulation, and the occurrence of oestrus (day 0) was checked every 8 hours using vasectomized rams. All ewes were hand-mated with intact rams. On day 5 after oestrus, ewes were anaesthetized, using an i.m. administration of 0.4 mL 2% xylazine and an *i.v.* injection of 10 mL sodium thiopental (20 mg mL^{-1}) (Thiobarbital, Braun Medical, Jaen, Spain). The reproductive tract was exposed by mid-ventral laparotomy and the number and side of CL were recorded. Uterine horns were then flushed with phosphate-buffered saline and pregnancy was defined as the presence of an embryo. After embryo recovery, ewes

were euthanized by injection of T-61 (Intervet, Salamanca, Spain) and both oviducts and samples of the middle of the upper one-third of each uterine horn were collected, frozen in liquid nitrogen and stored at –80°C until assayed. From the 14 ewes, 7 (4 control and 3 undernourished) presented CL only in one ovary; thus, the oviducts and uterine horns of these ewes were coded as ipsilateral or contralateral to the CL in order to study the local effect of the CL on gene expression.

RNA isolation and reverse transcription

Total RNA from uterus and oviducts was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) followed by precipitation with lithium chloride to remove inhibitors of cDNA synthesis and by DNase-treatment with DNA-Free™ Kit (Ambion, Austin, TX, USA) to remove contaminating DNA. Concentration of RNA was determined by measuring absorbance at 260 nm, the purity of all RNA isolates was assessed from 260 to 280 absorbance ratio and the integrity by electrophoresis (1% agarose gel). For each sample, cDNA was synthesized by reverse transcription using the Super-Script III transcriptase (Invitrogen) with oligo dT primers and 1 μg total RNA added as a template.

Quantitative real time PCR

Primers to amplify cDNA of the target genes *ESR1, PGR, IGF1* and *IGF2,* and of the endogenous controls

β-actin (*ACTB*) and ribosomal protein (*RP*) *L19* were used. Primer sequences and the expected product lengths are presented in Table 1. Real time PCR reactions were performed using 7.5 μL SYBR® Green mastermix (Quantimix EASY SYG kit, Biotools B&M Labs, Madrid, Spain), equimolar amounts of forward and reverse primers (200 nM, Operon Biotechnologies GmbH, Cologne, Germany), and 3 μL diluted cDNA (1:7.5 in RNase/DNase free water) in a final volume of 15 μL.

Samples were analyzed in duplicate in a 72-disk Rotor-Gene™ 6000 (Corbett Life Sciences, Sydney, Australia). Standard amplification conditions were 3 min at 95°C and 40 cycles of 15 s at 95°C, 40 s at 60°C, and 10 s at 72°C. At the end of each run, dissociation curves (melting curves) were analyzed to ensure that the desired amplicon was being detected and to discard contaminating DNA or primer dimers. Samples of cDNA of six ewes (three of each treatment) were pooled to provide an exogenous control and dilutions (n = 5 dilutions, from 100 to 6.25 ng tube^{-1}) of this pool were performed in uterus and oviduct. Linear regression of the dilution cDNA curves was performed for each gene and tissue and the efficiency (*E*) of the assays were calculated according to the formula $\%E = (10^{-1/\text{slope}} - 1) \cdot 100$ (Table 1) (Rutledge & Cote, 2003). For relative quantification, target gene expression was normalized to the mean expression of the endogenous control genes (*ACTB* and *RPL19*), taking into account the respective efficiencies (Pflaffl, 2009). Both *ACTB* and *RPL19* have been used before as endogenous controls in oviduct and/or uterus (Chen *et al*., 2006)

Table 1. Oligonucleotide primer sequence used (F: forward; R: reverse) for quantification of target and endogenous control gene cDNA

Gene	Accession No.	Primer sequence	Length (bp)	Efficiency uterus	Efficiency oviduct	References
PGR	Z66555	F: GACAGCACTTTCTAGGCGACAT R: TGTGCTGGAAGAAACGATTGC	79	0.92	1.20	Sosa *et al.* (2009)
ESR1	AYO33393	F: AGGGAAGCTCCTATTTGCTCC R: CGGTGGATGTGGTCCTTCTCT	234	1.02	1.30	Sosa *et al.* (2009)
IGF1	NM_001077828	F: CCAGACAGGAATCGTGGATG R: ACTTGGCGGGCTTGAGAG	89	0.79	1.15	Wu *et al.* (2004)
IGF2	AF106965	F: GACCGCGGCTTCTACTTCAG R: AAGAACTTGCCCACG	205	1.02	1.04	Pflaffl *et al.* (2002)
ACTB	U08283	F: CGAGCACGATGAAGATCAAGTT R: CCTCCGATCCACACCGAGTA	64	1.26	1.30	Chen *et al.* (2006)
RPL-19	AY158223	F: CCCCAATGAGGACCAATGAAATC R: CAGCCCATCTTTGATCAGCTT	119	1.03	1.00	Chen *et al.* (2006)

and remained unchanged among samples in this study. The intra-assay coefficient of variation (CV) for uterus was 6.5% and for oviduct was 8.6%.

Statistical analysis

Data were analyzed in a complete randomized design. All variables were subjected to analysis of variance using a mixed procedure that included in the model the nutritional treatment (low or control), organ (oviduct or uterus) and their interaction, and side of oviduct/uterus regarding CL (ipsi or contralateral) within the organ as fixed effects, and pregnancy as random effect. To analyze the local effect of the CL on gene oviductal and uterine expression, only ewes that presented CL in only one ovary were considered (n = 7); the statistical model included the organ (uterus/oviduct) and the side regarding the CL (ipsi or contralateral) within the organ as fixed effects, and both the nutritional treatment and pregnancy as random effects. Tukey-Kramer tests were conducted to analyze differences between groups. Data are presented as least square means ± pooled standard errors. Means were considered different when $p < 0.05$, and tendency to differ when $0.05 < p \leq 0.10$.

Results

Body weight and body condition score

Low group showed a decrease in body weight from day −15 to day 5 (61.1 ± 2.5 kg to 55.9 ± 2.5 kg, $p < 0.01$) whereas C group presented a slight increase (63.0 ± 2.5 kg to 64.4 ± 2.5 kg, $p < 0.05$). Low group tended to have lesser BCS than C group on day 5 (2.7 ± 0.1 and 2.9 ± 0.1, respectively, $p < 0.09$).

Ovulation and pregnancy rates

Ovulation rate (number of CLs per ewe) was not different between C and L groups (1.7 ± 0.5 and 2.4 ± 1.4, respectively).

Pregnancy rates were not statistically different between groups: 71.4% (5 out of 7) for C ewes and 42.9% (3 out of 7) for L ewes. The number of embryos recovered tended to be greater in C than in L ewes (1.3 ± 0.4 and 0.4 ± 0.2, respectively, $p = 0.06$).

Effects of undernutrition and organ

Expression of *PGR* was neither affected by organ nor nutritional treatment. There was an interaction between nutritional treatment and organ ($p < 0.01$). Low group presented higher *PGR* expression than the C group in oviducts. No differences were observed in uterus (Fig. 1a).

The expression of *ESR1* was not affected by organ, nutritional treatment or their interaction (Fig. 1b).

Expression of *IGF1* was unaffected by nutritional treatment, but both the effects of organ and the interaction between nutritional treatment and organ were significant ($p < 0.05$). A lower *IGF1* mRNA expression was found in the oviduct than in the uterus (1.66 ± 0.17 *vs.* 2.1 ± 0.17, $p < 0.05$) (Fig. 1c). Control ewes presented higher uterine expression of *IGF1* mRNA than L ewes ($p < 0.05$), but this did not occur in the oviducts (Fig. 1c).

Expression of *IGF2* was affected by organ ($p < 0.01$) and there was a tendency for the nutritional treatment ($p = 0.09$). No differences were observed for the interaction between nutritional treatment and organ. Overall, L ewes tended to present lower *IGF2* mRNA expression than C ewes (1.31 ± 0.19 *vs.* 1.78 ± 0.19, respectively, $p = 0.09$) and the oviduct presented greater expression than the uterus (1.89 ± 0.19 *vs.* 1.20 ± 0.19, $p < 0.01$). When Tukey-Kramer analyses were performed differences among treatments were found only in the oviduct but no in the uterus. Control ewes presented greater *IGF2* expression than L ewes in the oviducts, but no differences were found in the uterus (Fig. 1d).

Local effect of the corpus luteum

There was an effect of the laterality of the CL (contralateral *vs.* ipsilateral) in relation to the organ (oviduct/uterus) on *PGR* ($p < 0.05$), *ESR1* ($p < 0.03$) and *IGF2* ($p < 0.005$) expression (Fig. 2). While no differences were found in gene expression among the uterine horns, the contralateral oviduct presented greater *PGR*, *ESR1* and *IGF2* expression than the ipsilateral oviduct. Moreover, the contralateral oviducts presented greater expression of *ESR1* and *IGF2* than the contralateral uterine horns. No differences of side were found in *IGF1* mRNA expression (Fig. 2c).

Discussion

The present study demonstrates that undernutrition affects *PGR*, *IGF1* and *IGF*2 expression in an organ-
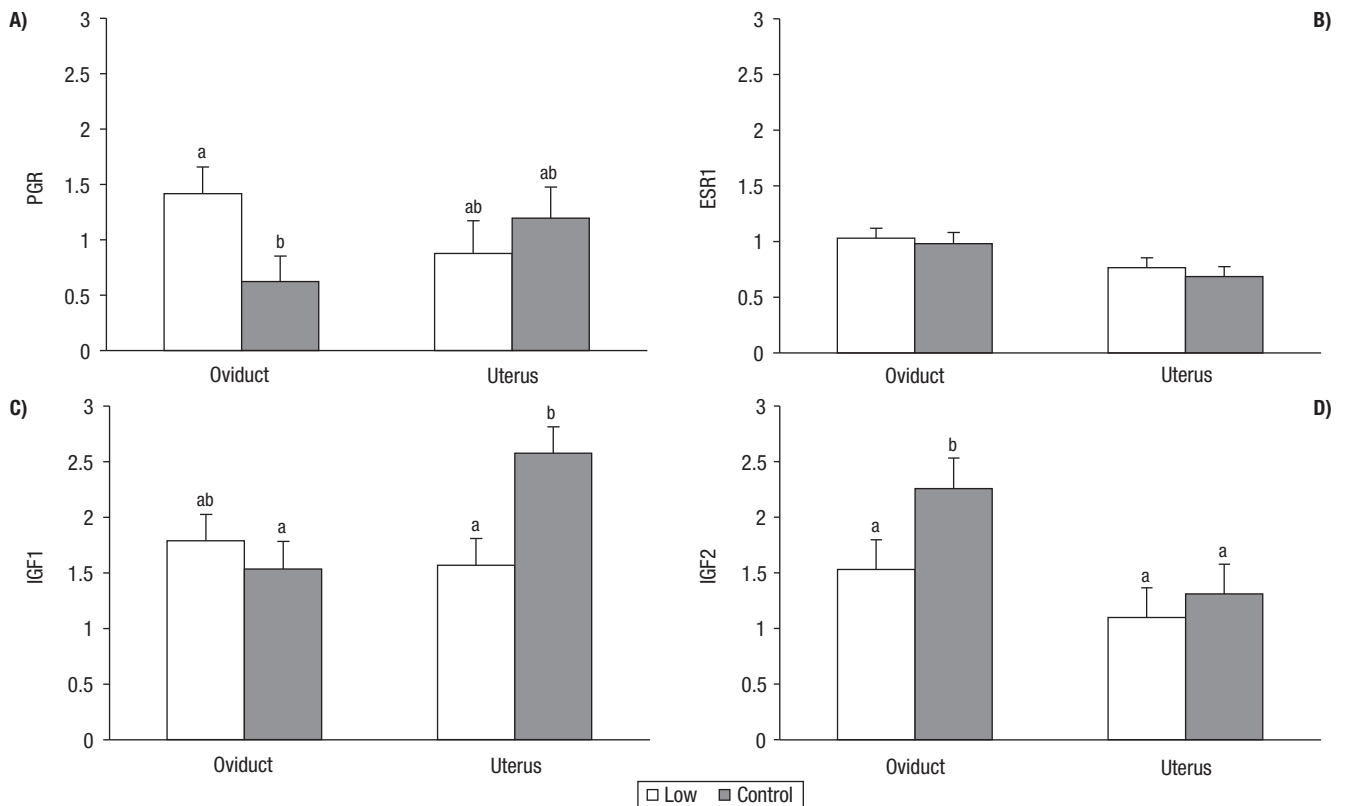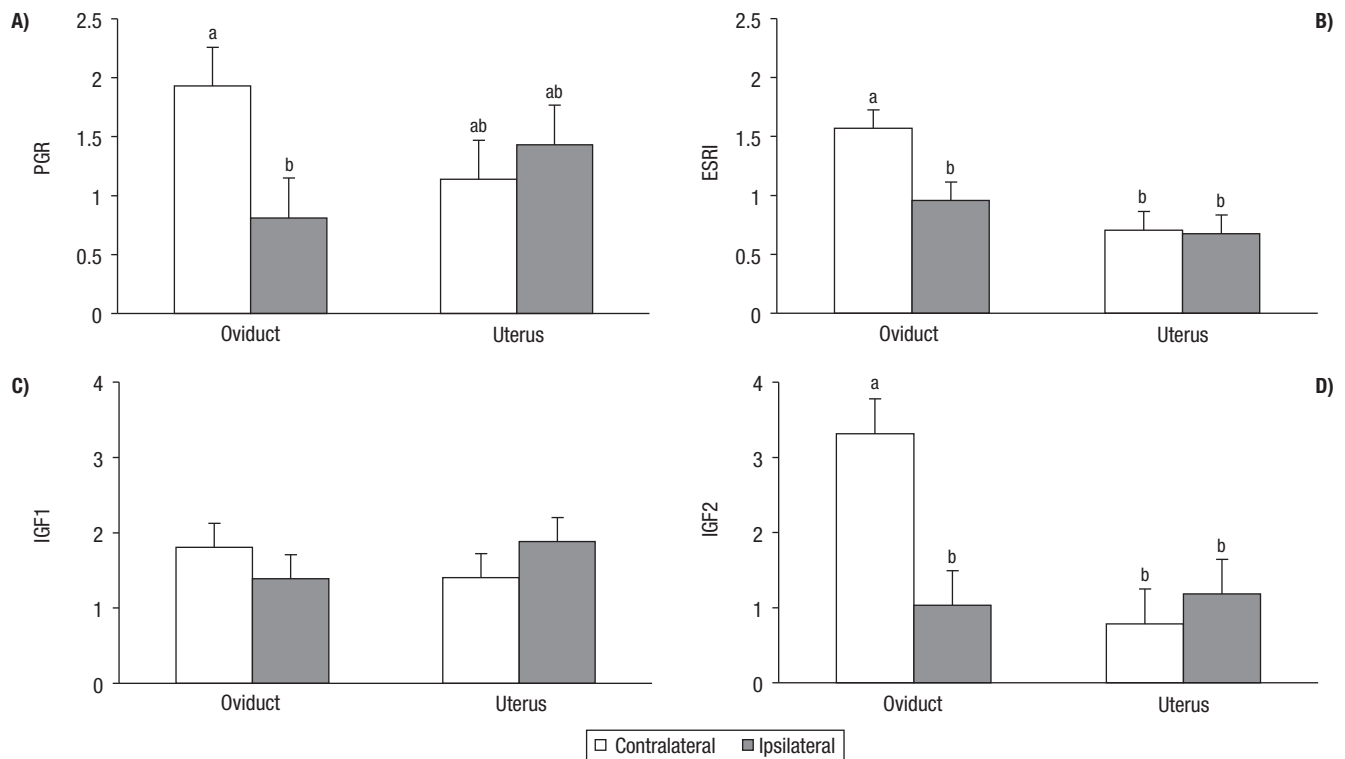
Figure 1. Relative expression of *PGR* (A), *ESR1* (B), *IGF1* (C) and *IGF2* (D) transcripts in oviduct and uterus of ewes fed 0.5 (Low, n = 7) or 1.5 (Control, n = 7) times the maintenance requirements on day 5 post-oestrus. Data are presented as least square means ± standard errors. Different superscripts (a,b) within the same graph indicate significant differences, $p < 0.05$.

dependent manner. Moreover, the side of the reproductive tract regarding the CL-bearing ovary on day 5 after oestrus affects *PGR, ESR1* and *IGF2* gene expression in the oviducts, but not in the uterus.

The oviducts accommodate gametes and early embryos until around day 5 after oestrus, when they enter the uterus to continue pregnancy. Before implantation, embryos develop unattached and depend on oviductal and uterine secretions (Ashworth, 1995), which in turn are regulated by steroid hormones and numerous growth and other regulatory factors (Geisert *et al.*, 1992). In the present study, oviducts presented a greater *IGF2* mRNA expression than the uterus, while the opposite occurred with *IGF1* expression. No differences between organs were found for the gene expression of sex steroid receptors. To our knowledge, there are no works designed to compare steroid receptors and growth factors expression between organs. Both *IGF1* and *IGF2* are implicated as regulators of pre-implantation and placental development. They stimulate the development of blastocysts cultured *in vitro* (Kaye *et al.*, 1992) and it is also thought they are important for

fetal development (Wathes *et al.*, 1998). Uterine derived growth factors are believed to modulate conceptus growth through paracrine pathways (Ashworth, 1995) and it has been suggested that IGF2 may have a more predominant role at these very early stages (day 5) of embryo development (Kaye, 1997), consistent with the greater expression of *IGF2* in the oviducts observed in the present study. On the other hand, IGF2 produced locally in the oviducts can also have an autocrine action since it may enhance the metabolic pathways necessary for the motility of the oviductal wall (Wathes *et al.*, 1998). IGF1 promotes embryo growth and development, but in addition, IGFs play a crucial role preparing an uterine environment capable of sustaining embryonic survival and implantation (Stevenson *et al.*, 1994). Our data suggest a major role of IGF2 in the oviducts and of IGF1 in the uterus. This is further supported by the effects of undernutrition on these factors in a specific organ dependent manner.

Greater *PGR* expression was found in the oviducts of undernourished ewes, which is in contrast to previous work from our group in cyclic ewes, using diffe-

Figure 2. Relative expression of *PGR* (A), *ESR1* (B), *IGF1* (C) and *IGF2* (D) transcripts in oviduct and uterus ipsilateral or contralateral to the corpus luteum (n = 7) on day 5 post-oestrus. Data are presented as least square means±standard errors. Different superscripts (a,b) within the same graph indicate significant differences, $p < 0.05$.

rent methodology (Sosa *et al.,* 2006). The greater PGR expression in undernourished ewes could be the result of a diminished P_4 down-regulation of its own receptor, since a lower PGR receptor content has been consistently reported (Sosa *et al.,* 2006; 2008).

Undernutrition affected the expression of the growth factors as undernourished ewes presented a lower gene expression of *IGF1* in uterus and of *IGF2* in oviducts. Feeding below the requirements for maintenance is known to cause embryo losses and retarded development of the embryo in sheep (Abecia *et al.,* 2006). This is consistent with the trend for fewer embryos in undernourished ewes in the present study. The altered gene expression where the embryo will be hosted could compromise embryo development due to the pivotal role of IGFs in mammalian embryo and fetal development (Pantaleon *et al.*, 2003). The greater expression of *IGF1* in the uterine horn of control ewes could increase embryo cell proliferation and prevent embryo losses exerting a positive effect on embryo development. Moreover, IGF1 is involved in protein increase and nutrient secretion from the endometrium that occurs immediately after oestrus in response to pro-

estrous oestrogen (Miller *et al.*, 1977). On the other hand, as *IGF2* mRNA is principally located in the muscle wall (Wathes *et al.*, 1998), the lesser expression of *IGF2* transcript found in the oviducts of undernourished ewes could alter the motility and prevent the embryo to be transferred to the uterus.

Even if the number of animals in the present experiment is limited, the expression of *PGR*, *ESR1* and *IGF2* was lower in the ipsilateral than in the contralateral oviduct, whereas no effects were observed in the uterus. This side-dependent expression probably relates to the different hormonal milieu established by the local distribution of ovarian products to the ipsilateral reproductive tract (Einer-Jensen & McCracken, 1981; Weems *et al.*, 1989; Wijayagunawardane *et al*., 1997). As P_4 modulates the action of oestrogens and its own action by a rapid reduction of ESR1 and PGR concentrations (Evans *et al.*, 1980), the lower level of *ESR1* and *PGR* mRNA found in the ipsilateral oviduct, could be due to the high local P_4 concentrations. Transcript levels of *IGF2* were also lower in the ipsilateral oviduct pointing to a similar regulation as *PGR* and *ESR1* mRNA, although regulation by local factors has

also been suggested (Stevenson & Wathes, 1996). These changes in gene expression may be important for the modulation of oviductal contraction and secretion, acting directly or interacting with several other genes expressed differently in the ipsilateral and contralateral oviducts (Bauersachs *et al*., 2003).

In conclusion, the present study reports that undernutrition decreases *IGF1* and *IGF2* gene expression in an organ-dependent manner. In addition, we report for the first time that growth factors and sex steroid receptors expression on day 5 after oestrus vary depending on the side of CL-bearing ovary and the region of the reproductive tract.

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