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 IGF2 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 (P₄) 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 P₄ concentrations (Abecia et al., 1996; Rhind et al., 1985). However, Lozano et al. (1998) demonstrated a reduction of P₄ 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 oviduct, 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 (± 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 groups 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

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**V. de Brun et al.** / **Span J Agric Res** (2013) 11(4): 989-996
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 SuperScript 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 $%E = (10^{-1/slope} - 1) \cdot 100$ (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: GACAGCAGTCTTTCTAGGCGACAT R: TGGTGGAGAAGAAACGATTGC  | 79          | 0.92             | 1.20               | Sosa et al. (2009)       |
| ESR1  | AYO33393      | F: AGGGAAGCTCCTATTTGCTCC R: CGGGGATGGTGGTCTTCTCT  | 234         | 1.02             | 1.30               | Sosa et al. (2009)       |
| IGF1  | NM_001077828  | F: CCAGACAGGAATCTGGATGAG R: ACTTGGGCCGGCTTGAAG  | 89          | 0.79             | 1.15               | Wu et al. (2004)         |
| IGF2  | AF106965      | F: GACGCCGCGTCTTTATCTTCAAG R: AAGACTGGGCCCCAG  | 205         | 1.02             | 1.04               | Pflaffl et al. (2002)    |
| ACTB  | U08283        | F: CGAGCCAGAAAGATCAAGTTTTT CCCTCGACTCAACCGAGTA  | 64          | 1.26             | 1.30               | Chen et al. (2006)       |
| RPL-19| AY158223      | F: CCCCAATAGGGACCAAATGAAATC R: CAGCCCATCTTTGATCGAG  | 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 \( IGF2 \) expression in an organ-
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-
rent methodology (Sosa et al., 2006). The greater PGR expression in undernourished ewes could be the result of a diminished P₄ 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-oestrous 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 P₄ 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 P₄ 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

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**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.
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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