PROGESTERONE SUPPLEMENTATION DURING THE PRE-IMPLANTATION PERIOD INFLUENCES INTERFERON-STIMULATED GENE EXPRESSION IN LACTATING DAIRY COWS*

Beatriz Serrano-Pérez^{1,2*}, Dimitrios Rizos³, Irene López-Helguera^{1,2}, Ester Molina¹,
Irina Garcia-Ispuerto^{1,2}, Fernando López-Gatius^{2,4}

¹Department of Animal Science and

²Agrotecnio Centre, University of Lleida, Lleida, Spain

³Department of Animal Reproduction, National Institute for Agriculture and Food Research
and Technology (INIA), Madrid, Spain

⁴Transfer in Bovine Reproduction SLU, Barbastro, Spain

*Corresponding author: bserrano@ca.udl.cat

Abstract

This study examined the effect of progesterone (P4) supplementation from Days 16 to 18 post-AI on interferon-stimulated gene (ISG) expression on Day 19 in high-producing dairy cows. Gene expression levels were measured in peripheral blood mononuclear cells. Possible relationships between ISG expression and the incidence of pregnancy failure were also investigated. Cows were alternately assigned on Day 16 post-AI to a control (C: $n = 13$) or treatment group (P4: $n = 14$). Out of 27 cows, 12 returned to oestrus before pregnancy diagnosis and 9 were diagnosed as pregnant on Day 28. ISG expression was assessed in all cows. Expression levels for the genes *OAS1*, *ISG15*, *MX1* and *MX2* were higher for pregnant than for non-pregnant cows ($P=0.04$; $P<0.001$; $P=0.02$; $P=0.045$; respectively). A significant ($P=0.01$) interaction was observed between the treatment and positive pregnancy diagnosis groups on Day 28 post-AI for the probability of showing ISG expression. This interaction suggests that in cows not pregnant on Day 28, P4 supplementation may have led to increased *ISG15* mRNA expression on Day 19. Lower *ISG15* expression was detected for cows returning to oestrus than for pregnant cows ($P<0.001$). However, cows with a negative pregnancy diagnosis showed intermediate values, differences being non-significant when compared to cows returning to oestrus or pregnant cows. Our results suggest that P4 supplementation during the pre-implantation period promotes conceptus signalling.

Key words: dairy cattle, fertility, maternal immune response, embryo mortality

Although in the last few decades a decline in fertility has paralleled increased milk yield in high-producing dairy cattle, such an increase may not be held respon-

*This work was financed by commercial dairy herds.

sible for this trend (Lucy, 2001; López-Gatius, 2003). In fact, the inability of cows to establish and maintain pregnancy may be the main limiting factor for the efficiency of dairy herds (López-Gatius, 2012). The embryonic period of gestation extends from conception to the end of the differentiation stage (about 45 days) and the foetal period spans from Day 45 to parturition (Committee on Bovine Reproductive Nomenclature, 1972). Most pregnancy losses occur during the first four weeks of pregnancy (Ayalon, 1978; Hansen, 2007). However, under intensive conditions of milk production, losses in the second month of pregnancy are increasing (López-Gatius and García-Ispuerto, 2010; López-Gatius and Hunter, 2017). During the pre-implantation period, luteal progesterone (P4) stimulates uterine receptivity for embryo recognition, implantation and subsequent development (Lonergan et al., 2016; Spencer et al., 2016). Secretion of interferon-tau (IFN- τ) by embryonic trophoblast cells favours corpus luteum maintenance (Mann and Lamming, 2001; Mann et al., 2006; Peter et al., 2017) and primes the maternal immune system to safeguard implantation success (Roberts et al., 1992; Spencer et al., 2007). This molecule affects both local (Binelli et al., 2001; Klein et al., 2006) and peripheral tissues (Gifford et al., 2007; Manjari et al., 2016; Matsuyama et al., 2012; Meyerholz et al., 2016; Ruhmann et al., 2017; Shirasuna et al., 2012).

The uterine micro-environment is a key factor promoting pregnancy (Bridges et al., 2013). In essence, poor embryo signalling during the recognition period may lead to pregnancy loss (Matsuyama et al., 2012; Wijma et al., 2016). Several therapeutic approaches have shown that exogenous P4 favours not only embryo development (Garrett et al., 1988; Carter et al., 2008) but also IFN- τ production (Mann et al., 2006). However, observed effects of P4 supplementation during this period on pregnancy maintenance have been variable, and reported effects of P4 treatment on the subsequent pregnancy rate have been positive (Friedman et al., 2012; Monteiro et al., 2014; García-Ispuerto et al., 2016; García-Ispuerto and López-Gatius, 2017), negative (Parr et al., 2014) or null (Monteiro et al., 2015). The factors proposed to affect the success of these therapies are period of treatment, artificial insemination (AI) method or previous reproductive disorders (Parr et al., 2014; Monteiro et al., 2014; García-Ispuerto et al., 2016).

On Day 19 of pregnancy, superficial implantation between the trophoblast and uterine epithelium begins (Guillomot et al., 1993; Peter et al., 2017). From Days 21–22 approximately, epithelial layers start to adhere by interdigitation of the microvilli (King et al., 1980, 1981). Conceptus viability during the peri-implantation period in ruminants can be assessed by characterising interferon-stimulated genes (ISG) in peripheral leukocytes (Han et al., 2006; Green et al., 2010; Matsuyama et al., 2012; Pugliesi et al., 2014; Kose et al., 2016; Alhussien et al., 2018). The objective of the present study was to examine ISG expression on Day 19 post-AI in peripheral blood mononuclear cells (PBMC) in lactating dairy cows supplemented with P4 from Days 16 to 18 post-AI. Possible relationships between gene expression and pregnancy failure were also explored.

Material and methods

Cattle and herd management

The study was performed on a high-producing Holstein-Friesian dairy herd in northeastern Spain. During the study period (May to October 2016), the herd was comprised of 625 lactating cows with a mean annual milk production of 12450 kg. The mean annual culling rate was 30%. Cows were grouped according to age (primiparous versus multiparous), milked three times daily and fed complete rations. All cows were subjected to a weekly reproductive health program, as described elsewhere (López-Gatius et al., 2006; García-Ispuerto and López-Gatius, 2014). Only healthy, cyclic, multiparous cows artificially inseminated (AI) following a five day P4-based fixed-time AI protocol (FTAI) (García-Ispuerto and López-Gatius, 2014) within 80 to 120 days in milk were included in the study. Further requirements for study inclusion were that cows were free of detectable reproductive disorders and of clinical diseases during the study period (Days 7 to 60 of insemination). A cow was considered to be cyclic when a mature corpus luteum was recorded at the beginning of the FTAI protocol.

Detection of oestrus, insemination, pregnancy diagnosis and pregnancy loss

Spontaneous oestrus was confirmed by rectal palpation in cows deemed to be in oestrus using a pedometer system, and AI was performed at this time. All cows were bred by AI using semen from bulls of proven fertility. If a cow returned to oestrus, its status was confirmed by rectal examination, and the animal was recorded as non-pregnant. In the remaining cows, pregnancy diagnosis was performed by ultrasound on Day 28 post-AI and confirmed on Day 56 post-AI. Since management and cow-related factors of a non-infectious nature have been extensively linked to late embryonic/early foetal loss in our geographical area (López-Gatius and García-Ispuerto, 2010; López-Gatius and Hunter, 2017), pregnancy loss was recorded when the 56-day diagnosis proved negative.

Experimental design

All procedures were approved by the Ethics Committee on Animal Experimentation of the University of Lleida (license numbers CEEA 09–01/12 and CEEA 09–01/13).

The selection criteria for the inclusion of cows in the experiment tried to favour the possibility of both pregnancy and pregnancy loss during the late embryo/early foetal period. Thus, only cows considered to be cyclic at the beginning of the FTAI protocol and with a single mature corpus luteum on Day 16 post-AI were selected. This selection increased the chances of pregnancy (García-Ispuerto and López-Gatius, 2014). Moreover, the study was performed during the warm period of the year (from May to October) in which pregnancy losses are greatly increased in our geographical area (López-Gatius and Hunter, 2017).

On Day 16 post-AI, cows were randomly assigned to a control, no-treatment group (C: $n=13$), or treatment group (P4: $n=14$). Animals in the P4 group were fitted with a P4-releasing intravaginal device (PRID DELTA, containing 1.55 g of P4;

CEVA Salud Animal, Barcelona, Spain) for 3 days. Cows were included only once in the experiment. The final data analysed corresponded to 27 multiparous cows.

Blood sampling

Blood samples were collected from each animal by tail vein puncture into EDTA vacuum tubes (BD Vacutainer™, Becton, Dickinson and Company, Plymouth, UK) on Days 16 and 18 post-AI for plasma P4 determination (4 mL), and on Day 19 post-AI for haematological analysis and isolation of PBMC (8 mL). Blood samples were placed immediately on ice and those for P4 analysis were centrifuged (10 min at 1,600 × g) within 30 min of collection and the plasma stored at -20°C until analysis.

Progesterone concentration assay

Progesterone concentrations were determined in plasma using a direct method (without extraction) as previously described in detail (López-Gatius et al., 2007). The minimum detection limit of the P4-RIA technique used was 0.15 ng/mL and intra-assay and inter-assay coefficients of variation were 5.1% and 10.5%, respectively.

Isolation of PBMC, RNA extraction and cDNA synthesis

Peripheral blood mononuclear cells were isolated by centrifugation on a Ficoll density gradient (Histopaque, Sigma, St Louis, MO) followed by repeated rinsing in phosphate buffered saline (PBS) solution. The isolated PBMCs were lysed in Trizol™ (Invitrogen Corp., Carlsbad, CA, USA) and kept at -80°C until RNA analysis. Total RNA was extracted according to the method of Chomczynski and Sacchi (1987). Concentrations of RNA were determined spectrophotometrically, and samples were treated with DNase in the presence of RNase inhibitors to eliminate contaminating genomic DNA. Complementary DNA was synthesized in a total volume of 20 µL, from 1 µg of total RNA in the presence of random primers and reverse transcriptase using the RevertAid H Minus First Strand cDNA synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's recommendations.

Quantitative Real-Time PCR

Messenger RNA expression was determined by Quantitative Real-Time PCR (qPCR) for four target genes – *interferon-stimulated gene 15 (ISG15)*, *20-50-oligoadenylate synthase 1 (OAS1)*, *myxovirus resistance 1 (MX1)*, and *myxovirus resistance 2 (MX2)* – and two reference genes – *β-actin (ACTB)* and *ribosomal protein L19 (RPL19)* (Table 1). To avoid genomic contamination, all primers were selected to span an intron. For each gene, we generated a standard curve by amplifying serial dilutions of a control cDNA to check for linearity between initial template concentration and cycle threshold (Ct) values. Amplifications were conducted using the SYBRgreen method with the ABI PRISM™ 7500 sequence detector (Applied Biosystem, Foster City, CA, USA) following the manufacturer's recommendations: an initial activation and denaturation step of 10 min at 95°C followed by 40 cycles consisting of 10 s at 95°C and 1 min at 60°C. PCR reactions were run using 3 µL of 30-fold diluted cDNA as template in a total volume of 8 µL containing 1× Maxima

SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, USA), 200 nM of forward and reverse primers as published elsewhere (Serrano-Pérez et al., 2016). Each measurement was carried out in triplicate and the average used to calculate the relative gene amount. Data normalization and analysis were performed by the $\Delta\Delta C_t$ method (Yuan et al., 2006) using the mean C_t values from the two reference genes and the C_t value for each ISG.

Table 1. Sequence, NCBI sequence and reference of the primers used for quantitative PCR

Gene		Sequence (5' – 3')	NCBI sequence	Reference
<i>ISG15</i>	Fwd	CGCAGCCAACCAGTGTCT	NM_174366.1	Paradis et al., 2015
	Rev	CGTCATGGAGTCCCTCAGA		
<i>OAS1</i>	Fwd	TCATCCGCCTGGTGAAGCACTGG	NM_174366.1	Manjari et al., 2016
	Rev	TTGCTCCCAGGCATAGACCGTCAG		
<i>MX1</i>	Fwd	GTACGAGCCGAGTTCTCAA	AF047692	Ribeiro et al., 2014
	Rev	ATGTCCACAGCAGGCTCTTC		
<i>MX2</i>	Fwd	CTTCAGAGACGCCTCAGTCG	NM_173941	Ribeiro et al., 2014
	Rev	TGAAGCAGCCAGGAATAGTG		
<i>ACTB</i>	Fwd	CTGGACTTCGAGCAGGAGAT	AY141970	Monteiro et al., 2014
	Rev	GATGTGCACGTCACACTTC		
<i>RPL19</i>	Fwd	GATCCGGAAGCTGATCAAAG	NM_001040516.1	Serrano-Pérez et al., 2016
	Rev	ATTGAGCATTGGCAGTACC		

Data collection and statistical analysis

The following data were recorded for each animal: parturition and AI dates; lactation number; milk production at AI; treatment (control vs. P4); return to oestrus, conception or no conception on Day 28 post-AI; P4 concentrations on Days 16 and 18 post-AI; and *ISG15*, *OAS15*, *MX1* and *MX2* gene expression in PBMCs on Day 19 post-AI. Fixed effects included in the models were treatment, return to oestrus, pregnancy and plausible interactions. The possible effects of variables on P4 concentrations on Days 16 and 18 were analysed by GLM repeated measures analysis of variance using the SPSS computer package, version 17.0 (SPSS Inc., Chicago, IL). The Student's t-test or one-way ANOVA test were used to compare relative ISG gene expression for fixed effects and their interactions. When significant differences were detected, the Bonferroni test was used to examine all possible pairwise comparisons. The level of significance was set at $P < 0.05$. Data are provided as the mean $\pm$ standard error of the mean (SEM).

Results

Mean values of lactation number and milk production at AI were 3.4 ± 1.3 lactations (range 2–7 lactations) and 46.9 ± 9.5 kg (range 35–85 kg), respectively. Of the

27 cows enrolled, 12 (44.4%) returned to oestrus between Day 19 to 23 post-AI. Out of the 15 (55.6%) cows not returning to oestrus, 9 were diagnosed as pregnant on Day 28 post-AI (5 C and 4 P4 cows), while 6 were negative (1 C and 5 P4 cows). Pregnancy loss was not recorded in any cow on Day 56 post-AI.

Progesterone concentrations on Day 18 were significantly higher ($P=0.03$, within subject effects) in P4 cows compared to C cows (Figure 1). There was no significant interaction between treatment group and pregnancy for plasma P4 concentrations.

Figure 1. Mean plasma P4 concentrations (units $\pm$ SEM) recorded on Days 16 and 18 post-AI in the control (n=13) and P4 group (n=14). Differences on Day 18 were significant ($P=0.03$, within subject effect, by GLM repeated measures analysis of variance)

Figure 2. Interaction between treatment group and pregnancy for mean plasma P4 concentrations (units $\pm$ SEM) recorded on Days 16 and 18 post-AI

Figure 3. Relative fold differences in *OASI*, *ISG15*, *MX1* and *MX2* gene expression according to the interaction between the treatment group and pregnancy on Day 28. The non-pregnant CONTROL group was designated as calibrator to compare expression levels. Bars represent mean values $\pm$ SEM. a, b – values differed among groups according to the Bonferroni test ($P=0.01$)

Figure 4. Relative fold differences in *OASI*, *ISG15*, *MX1* and *MX2* gene expression in PBMCs on Day 19 post-AI for cows returning to oestrus ($n=12$), cows not returning to oestrus ($n=6$) and pregnant cows ($n=9$). Group of cows returning to oestrus was designated as calibrator to compare expression levels.

Bars represent mean values $\pm$ SEM.

a, b – values differed among groups according to the Bonferroni test ($P<0.05$): a vs b, $P<0.001$; a vs ab, $P=0.075$; ab vs b, $P=0.126$

Interferon-tau stimulated gene expression was recorded in all cows. The expression of *OASI*, *ISG15*, *MX1* and *MX2* was higher for pregnant than non-pregnant cows (Student's t-test; $P=0.04$; $P<0.001$; $P=0.02$; $P=0.045$; respectively). There was a significant interaction between treatment group and pregnancy for the probability of ISG expression (Bonferroni test: $P=0.01$; Figure 3). This interaction implies that in cows not pregnant on Day 28, P4 supplementation could be increasing *ISG15* mRNA expression 2.4-fold on Day 19 as compared to not pregnant control dams. Lower *ISG15* mRNA expression values were observed for cows returning to oestrus than those maintaining gestation ($P<0.001$). However, not pregnant cows that did not return to oestrus before Day 28 showed intermediate *ISG15* expression values, with no differences when compared to cows returning to oestrus ($P=0.075$) or pregnant cows ($P=0.13$) (Bonferroni test; Figure 4).

Discussion

This study was designed to characterize embryo-maternal crosstalk during the peri-implantation period after P4 supplementation (Days 16 to 18 post-AI) by examining ISG mRNA expression in PBMCs on Day 19 post-AI. Our main findings were that: 1) P4 supplementation led to significantly increased *ISG15* expression levels on Day 19 post-AI in non-pregnant cows; and 2) cows returning to oestrus showed significantly lower *ISG15* levels than cows remaining pregnant, while cows not returning to oestrus showed intermediate *ISG15* levels with non-significant differences when compared to cows returning to oestrus or maintaining their pregnancy.

A large body of published work has demonstrated the pleiotropic effect of P4 in modulating the uterine environment, histotroph secretion and embryo development (Clemente et al., 2009; Forde et al., 2010, 2012). However, the effect of exogenous P4 treatment during the peri-implantation period on fertility remains unknown (Stevenson et al., 2007; Monteiro et al., 2015). Strategies for optimising maternal recognition often fail to improve embryo survival probably as a consequence of intrinsic factors in the embryo (Loneragan et al., 2016) or of a compromised uterine environment (López-Gatius et al., 1996; García-Ispuerto et al., 2016; Ribeiro et al., 2016). In addition, duration and term of P4 treatment also influences the reproductive performance in dairy herds. For example, a two week P4 treatment in the early luteal phase (Days 5–19) reduced luteal vascularization and plasma P4 secretion (García-Ispuerto and López-Gatius, 2012). In contrast, a shorter treatment during the pre-implantation period at Days 12–16 did not improve embryo development and IFN- τ production (Mann et al., 2006). In a recent study (García-Ispuerto et al., 2016), we noted the efficacy of P4 supplementation during the time of pregnancy recognition in cows without a clinical history of placenta retention. Accordingly, exogenous P4 treatment in healthy, cyclic cows from Days 16 to 18 post-AI was found to increase not only plasma P4 concentrations but also *ISG15* expression in maternal PBMC on Day 19, even in cows that were not pregnant on Day 28. Thus, P4 supplementation might favour embryo signalling in all cows, although some embryos were ultimately

lost. The potential benefits of P4 supplementation on embryo elongation have been linked to changes in the transcriptional response of the endometrium more than the embryo itself (Forde et al., 2009, 2010). We infer that P4 treatment may enhance secretions of the glandular uterine epithelium favouring conceptus elongation and IFN- τ secretion. The finding that P4 supplementation can lead to increased *ISG15* expression in cows suffering embryo loss is valuable. This may suggest an improvement in endometrial physiology that influences positively embryo growth and IFN- τ secretion in dams with a compromised uterine environment. However, in agreement with other studies (Matsuyama et al., 2012; Wijma et al., 2016), conceptus growth does not necessarily imply pregnancy establishment. Cows not returning to oestrus showed intermediate IFN- τ signalling levels compared to pregnant cows and to cows returning to oestrus. Impaired embryos seem to induce *ISG15* mRNA expression in peripheral tissues during the maternal recognition period in spite of their later demise (Han et al., 2006; Matsuyama et al., 2012; Kose et al., 2016; Wijma et al., 2016). Negative pregnancy following P4 treatment from Days 16 to 18 might be a consequence of poor stimulation of the endometrial transcriptome or of non-infectious factors affecting fertility after the period of recognition of pregnancy (López-Gatius, 2012). In a previous study, higher efficacy of a short treatment with P4 given in the early luteal phase (Days 3 to 5 post AI) was observed (García-Ispuerto and López-Gatius, 2017), in agreement with the findings of a meta-analysis by Yan et al. (2016). In our study, all losses were probably due to heat stress conditions. Management practices related to high milk production and fertility seemed here not to overcome the negative effects of heat stress on the embryo, uterine microenvironment or both.

As an overall conclusion, our results suggest that P4 supplementation during the pre-implantation period influences conceptus signalling. Further work is needed to examine the potential effects of heat stress on embryo signalling.

Acknowledgements

The authors thank Ana Burton and Dr. Sylvia J. Bedford-Guaus for assistance with the English translation, the owner and staff of Allué farm for the use of their cows and facilities, Professor J.F. Beckers and N.M. de Sousa for P4 determinations, Tarini Tuono for her assistance in sampling and PCR analysis, and Romi Pena for her valuable advice in laboratory performance.

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