

## **EFFECT OF PREGNANCY ON ENDOMETRIAL SEX STEROID RECEPTORS AND ON PROSTAGLANDIN F<sub>2α</sub> RELEASE AFTER UTERINE BIOPSY IN HEIFERS**

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**Abstract:** The effect of pregnancy on oestrogen receptor (ER) and progesterone receptor (PR) endometrial expression in heifers was studied. Holstein heifers were not inseminated (controls, n = 8) or inseminated (n = 21). Endometrial biopsies were taken at Day 17 from the uterine horn ipsilateral to the corpus luteum. Hourly samples were taken on the day of the biopsy in 12 animals (controls = 4 and inseminated = 8) to analyze 15-ketodihydro-PGF<sub>2α</sub> (PGFM) and progesterone concentrations. Pregnancy determined by ultrasonography diagnosed 6 pregnant cows. The uterine biopsy increased PGFM concentrations, which remained high for 2 to 4 hours, followed by a transient decrease in progesterone concentrations, but the procedure neither provoked luteolysis nor blocked pregnancy. PGFM concentrations were higher in cyclic than in pregnant cows. No differences in PR mRNA expression were observed among groups, but ER mRNA in pregnant heifers tended to be lower than controls, suggesting that this pathway is implicated in maintenance of pregnancy.

### **1. INTRODUCTION**

Genetic improvement for milk production during the last decades has been associated with decreased reproductive efficiency (Lucy, 2001). Selection for reproductive efficiency in dairy cows has not been attempted worldwide, with the main causes being the time required to evaluate

reproductive parameters and the lack of appropriate markers for fertility. One important cause of reproductive failure has been attributed to embryo loss, which has been estimated to be approximately 40 percent (Thatcher *et al.*, 2003), that results from failures in maintaining the life of the corpus luteum. The process whereby the regression of the corpus luteum (luteolysis) is blocked in early gestation in ruminants has been termed maternal recognition of pregnancy (Short, 1969).

In order to inhibit luteolysis, the ruminant embryo delivers a signal (interferon- $\tau$  (IFN- $\tau$ )) along the uterine horns by elongation. The IFN- $\tau$ , acting by modifying uterine gene expression in pathways not completely understood, affects the episodic prostaglandin  $F_{2\alpha}$  (PGF $_{2\alpha}$ ) release that is responsible for the luteal regression. During this period, the embryo is free-living in the uterine lumen and is completely dependent on uterine secretion for all its metabolic needs. Oestrogen and progesterone, acting via their intracellular receptors, ER and PR, are the main regulators of uterine function and have been implicated in the control of luteolysis (Lamming *et al.*, 1995). Maximum concentrations of ER and PR were found around oestrus, while the lowest concentrations of receptors were found at dioestrus (Zelinski *et al.*, 1982; Vesanen *et al.*, 1988). In addition, differences in gene expression were found in heifers with short and normal cycle: cows with short cycle presented higher ER mRNA concentration at day 5 and lower PR mRNA concentration at day 12 (Meikle *et al.*, 2001). These results show that the endometrium of heifers with a short cycle has an altered gene expression during the early luteal phase, which cannot be explained by gene expression at day 0 or by the circulating concentration of steroids between days 0 and 5 (no differences between groups). Heifers that have short-lived corpora lutea and consequently a short cycle are not capable of maintaining pregnancy.

Although progesterone is the principal hormone implicated in the control of embryo development and IFN- $\tau$  secretion (Mann and Lamming, 1995), the role of PR in pregnancy remains unclear. Studies consistently report a loss of PR in the uterus around the time of luteolysis in both sheep and cattle (Spencer and Bazer, 1995; Mann *et al.*, 1999; Robinson *et al.*, 2001). These findings are puzzling if we consider the importance of this hormone around the time of maternal recognition of pregnancy. Regarding ER, different responses have been reported in early pregnancy in cattle, such as no changes (Robinson *et al.*, 1999), a decrease in expression within all layers of the endometrium at days 16 and 18 of pregnancy (Robinson *et al.*, 2001), or an increase in uterine glands and stroma but a decrease in luminal epithelium (Kimmins and MacLaren, 2001).

In this study we focused on the effect of the embryo on gene endometrial expression (embryo-to-mother signalling) on sex steroid receptor endometrial expression. As a first step, we investigated if uterine biopsy in heifers

provokes PGF<sub>2α</sub> release, and if it also induces luteolysis or allows pregnancy to be maintained.

## **2. MATERIALS AND METHODS**

### **2.1 Experimental design**

The experiment was carried out at the INIA experimental farm, Colonia, Uruguay. Twenty-nine Holstein heifers in heat (day 0) were selected after synchronization with two injections of an analogue of PGF<sub>2α</sub> at an interval of 12 days. Only animals with normal cycle length were analysed. The animals had (mean ±SE) an age of 20.3 ±0.9 months, a body weight of 358 ±8 kg and a body condition score of 2.25 ±0.1 (on a scale of 1 to 5). Eight cows were kept as control and not inseminated, while 21 were inseminated 12 hours after showing standing oestrus. Endometrial biopsies were taken at day 17 from the uterine horn ipsilateral to the corpus luteum, and tissue samples were frozen immediately in liquid nitrogen and stored in a freezer at -80°C until the analysis. Special care was taken in collecting the endometrial samples in order to maintain pregnancy, and the amount of tissue sampled was approximately 0.1 g. Blood samples for progesterone determination were taken daily from day -1 to day +25. An hourly sampling was taken from 5 hours before to 12 hours after the biopsy in 12 animals (four from the control group and eight from the inseminated group) to analyse the PGF<sub>2α</sub> release by 15-ketodihydro-PGF<sub>2α</sub>. Pregnancy was determined by ultrasonography 35 days after oestrus, and animals were classified in three groups: control, and artificial inseminated (AI) non-pregnant (AI non-pregnant) and pregnant (AI pregnant) heifers.

### **2.2 Hormone determination**

#### **2.2.1 Progesterone**

Progesterone (P<sub>4</sub>) was determined in plasma using a commercial kit (Coat-a-count, DPC Diagnostic Products Co., Los Angeles CA, USA). In the plasma assay, the intra- and inter-coefficients of variation were 9 percent and 8 percent, respectively. The sensitivity of the assay was 0.04 nmol/litre.

### 2.2.2 15-Ketodihydro-PGF<sub>2α</sub>

The plasma metabolite of PGF<sub>2α</sub> was analysed in unextracted plasma by RIA. The detection limit of the assay was between 25 and 30 pmol/litre. The intra-assay coefficient of variation was below 11 percent and the inter-assay coefficient of variation was 14 percent.

### 2.2.3 Cortisol

Cortisol was determined using a solid phase RIA kit (Coat-a-count, DPC Diagnostic Products Co., Los Angeles CA, USA). The detection limit of the assay was 6 nmol/litre. All samples were determined in the same assay and the intra-assay coefficient of variation for control samples was below 8 percent.

## 2.3 mRNA determination

A solution hybridization assay of specific mRNAs for ER $\alpha$  and PR was performed in endometrial samples as described previously (Meikle *et al.*, 2001). In short, total nucleic acids (TNA) were prepared and the concentration of DNA in the TNA samples was measured fluorometrically. Probes were synthesized *in vitro* and radiolabelled with <sup>35</sup>S-UTP. The probes used for ER $\alpha$  mRNA and PR mRNA determinations were derived from full-length cDNAs containing the whole open reading frame of the human oestrogen and progesterone receptors, respectively. The cross-reactivity between bovine mRNA and human probes for ER $\alpha$  and PR has been demonstrated previously by northern blot (Meikle *et al.*, 2001). Overnight incubation was performed at two different concentrations, and samples were then treated with RNase to digest unhybridized RNA. Labelled hybrids were precipitated with trichloroacetic acid, collected on filters and the radioactivity was determined in a liquid scintillation counter. All the samples from the experiment were determined in the same assay. Receptor mRNA concentrations are expressed as counts per minute (cpm) in relation to DNA content (cpm/ $\mu$ g DNA).

## 2.4 Statistical analyses

Statistical analysis was carried out using the Statistical Analysis System (SAS Institute Inc., Cary NC, USA). Progesterone, cortisol and 15-ketodihydro-PGF<sub>2α</sub> concentrations were analysed by a mixed procedure (SAS) and the statistical model included the effects of group (control, AI non-pregnant and AI pregnant heifers), day and the interaction between

group and day, and also the random effect of cow within the group. Data of mRNA of ER and PR were analysed by orthogonal contrast using SAS.

### 3. RESULTS

#### 3.1 Effect of the uterine biopsy on 15-ketodihydro-PGF<sub>2α</sub>, progesterone and cortisol levels

Determination of 15-ketodihydro-PGF<sub>2α</sub>, progesterone and cortisol were performed in plasma samples collected from 5 hour before to 12 hour after the performance of the uterine biopsy on day 17 of the oestrous cycle in 12 heifers (controls  $n = 4$ ; AI  $n = 8$ ). The inseminated heifers were classified as pregnant ( $n = 3$ ) or non-pregnant ( $n = 5$ ) by ultrasound diagnosis at day 35. One AI non-pregnant heifer that had low concentration of P<sub>4</sub> on day 17 was already in luteolysis; this animal was excluded from the analyses. The uterine biopsy increased 15-ketodihydro-PGF<sub>2α</sub> concentration in the first bleeding after performance of the biopsy ( $p < 0.0001$ ), and concentration remained high for the following 2 to 4 hours (Figure 1).

Progesterone concentration increased in the first bleeding after the biopsy ( $p < 0.01$ ) but concentration then decreased 2 to 4 hours after the biopsy, consistent with the 15-ketodihydro-PGF<sub>2α</sub> peak observed (Figures 1 and 2). No statistical difference in P<sub>4</sub> concentration according to physiological status (pregnant vs non-pregnant) could be detected. The significant increase 1 hour after the biopsy was consistent with a cortisol peak at that moment (Figure 2). There was a significant correlation between the two hormones:  $r = 0.2079$  ( $p = 0.003$ ).

#### 3.2 Outcome of pregnancy

The oestrous cycle in control and inseminated non-pregnant heifers had a duration of  $20 \pm 0.3$  and  $20.8 \pm 1.2$  days (oestrus to oestrus). Progesterone concentrations from day 0 to day 25 post-oestrus in control (not inseminated), AI non-pregnant and AI pregnant heifers are shown in Figure 3. At day 35, 6 out of 21 inseminated heifers were diagnosed as pregnant. Inseminated non-pregnant cows ( $n = 15$ ) were classified in two groups according to P<sub>4</sub> concentrations at days 21 to 25: luteal (P<sub>4</sub> > 18 nmol/litre; AI non-pregnant A;  $n = 2$ ) or basal (P<sub>4</sub> < 3 nmol/litre; AI non-pregnant B;  $n = 13$ ). Heifers with luteal concentration of P<sub>4</sub> at day 25 (AI non-pregnant A) may have suffered early embryonic mortality and were possibly pregnant at day 17; they were therefore considered as pregnant for mRNA analysis (pregnant,  $n = 8$ ).

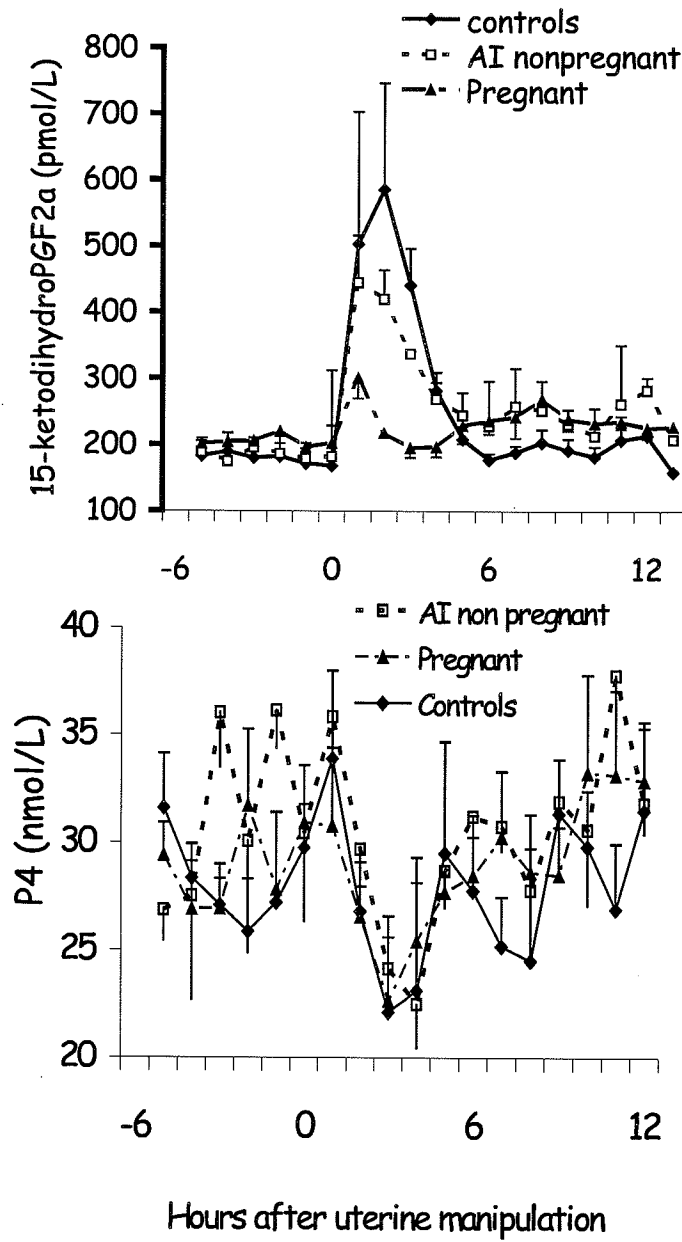


Figure 1. Mean ( $\pm$  standard error of the mean) concentrations of 15-ketodihydro-PGF<sub>2α</sub> (top panel) and progesterone (bottom panel) in control (n = 4), inseminated non-pregnant (n = 4) and pregnant (n = 3) heifers before and after uterine biopsy.

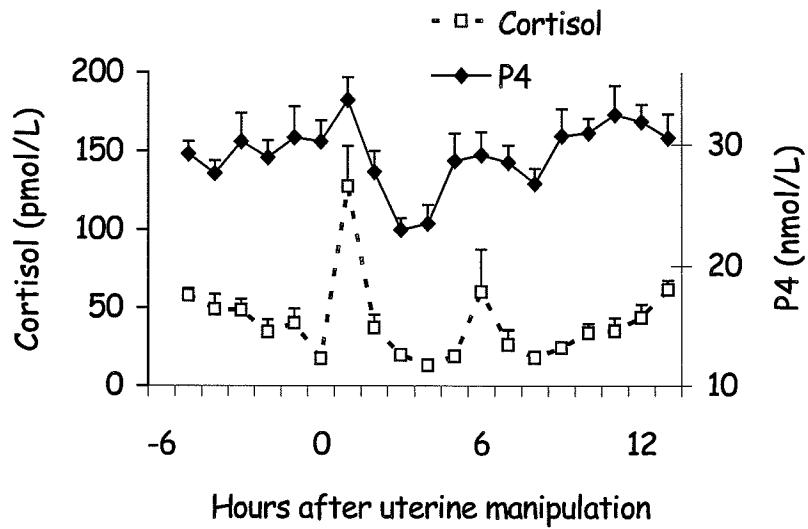


Figure 2. Mean ( $\pm$ SE) concentrations of progesterone and cortisol in heifers before and after uterine biopsy.

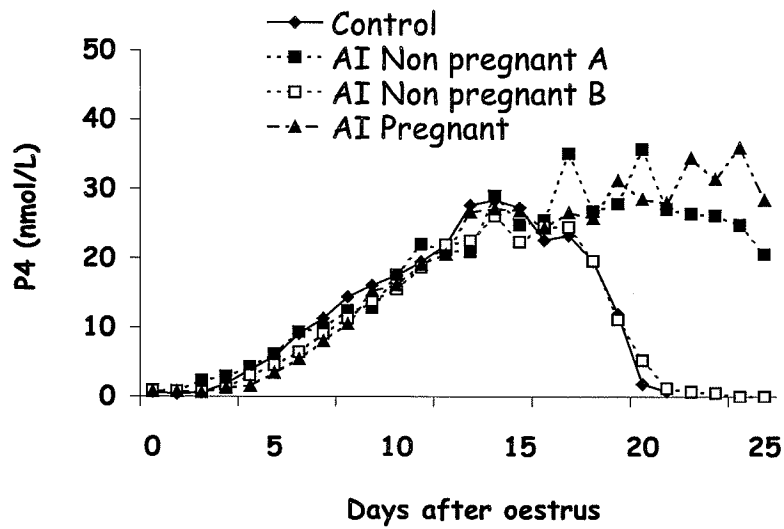


Figure 3. Mean concentrations of progesterone in control, pregnant, non-pregnant heifers with luteal (A) or basal (B) concentration of P4 at day 25 post-oestrus.

### 3.3 Effect of pregnancy on endometrial mRNA expression of ER and PR

The results of ER mRNA and PR mRNA are shown in Figure 4. No difference could be demonstrated in PR mRNA expression of the different groups. The concentration of ER mRNA in pregnant heifers tend to be lower than control ( $p < 0.1$ ).

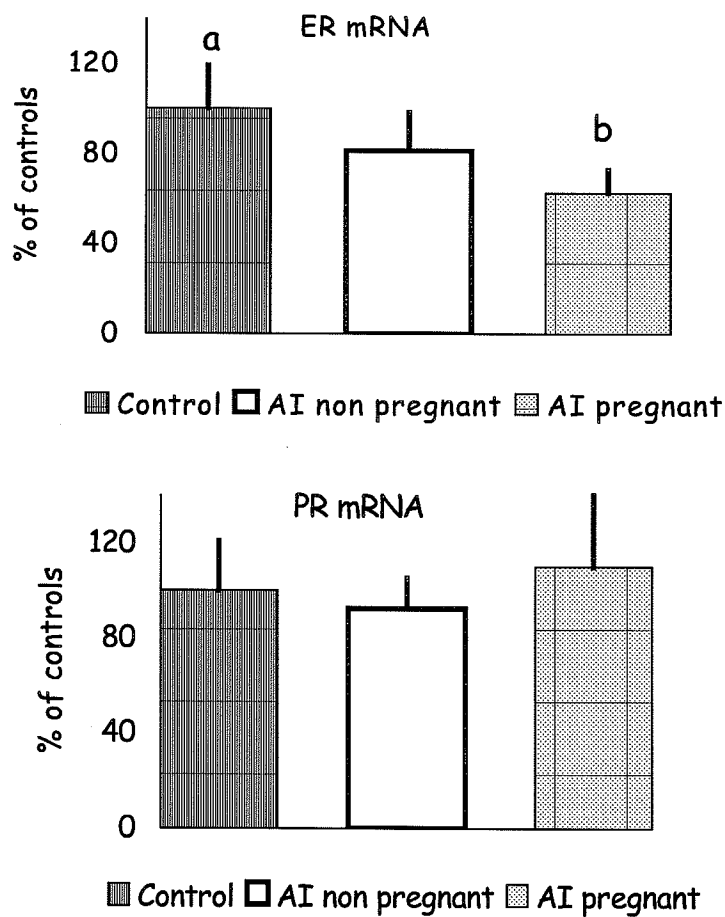


Figure 4. Concentrations of mRNA of oestrogen receptor  $\alpha$  (ER mRNA; top panel) and progesterone receptor (PR mRNA; bottom panel) in the endometrial biopsies on day 17 in control, AI non-pregnant and AI pregnant heifers. The results are presented as percentages of control heifers. Bars are least square mean  $\pm$ SE of a versus b,  $p < 0.10$ .



#### 4. DISCUSSION

This is the first study to describe that the effect of an endometrial biopsy on  $PGF_{2\alpha}$  release is dependent on the physiological status (pregnant vs cyclic heifers).

Measurement of  $PGF_{2\alpha}$  itself in the peripheral circulation is not a suitable parameter since it has an extremely short half-life; 15-ketodihydro- $PGF_{2\alpha}$ , a parent compound of  $PGF_{2\alpha}$  and having a longer half-life, has been widely used as a good parameter of  $PGF_{2\alpha}$  release (Basu *et al.*, 1987). The 3-fold increase of 15-ketodihydro- $PGF_{2\alpha}$  concentration after the uterine biopsy on day 17 is consistent with the known ability of the uterus to secrete high  $PGF_{2\alpha}$  concentrations during late dioestrus (Kindahl, Lindell and Edqvist, 1981). There was an effect of the physiological status on 15-ketodihydro- $PGF_{2\alpha}$  concentration; pregnant cows had lower concentration of 15-ketodihydro- $PGF_{2\alpha}$  than control and AI non-pregnant heifers 1 to 3 hour post biopsy. Similar results have been previously reported by Thatcher *et al.* (1995): the magnitude and frequency of  $PGF_{2\alpha}$  release from the uterus in non-pregnant cows are higher than that of pregnant cows. This agrees with the final action of the embryo for maternal recognition of pregnancy in ruminants: the embryo signal, interferon- $\tau$ , inhibits the episodic  $PGF_{2\alpha}$  secretion and in consequence the corpus luteum is maintained.

The uterine biopsy induces a temporary release of  $PGF_{2\alpha}$  which is followed by a transient decrease in progesterone concentration, but this procedure does not provoke luteolysis or block pregnancy. Pregnancy was maintained even after entering the horn ipsilateral to the corpus luteum by a transcervical catheter and performing an endometrial biopsy. Thus, transcervical biopsies can be used for studies on the interaction between the conceptus and maternal mRNA endometrial expression. This is useful, avoiding complicated surgery or expensive slaughter. This methodology may allow repeated measurements of gene uterine expression during a biological process.

The progesterone increase observed one hour after the biopsy was surprising, and therefore cortisol was determined. There was an important release of cortisol one hour after the biopsy, which may indicate an important stress for the animals. The correlation observed in progesterone and cortisol concentrations is of no surprise, since progesterone is one of the precursors of cortisol, and may explain the increase found in progesterone concentration one hour after the biopsy (Bolaños, Molina and Forsberg, 1997).

There was a tendency in pregnant heifers to have lower ER mRNA concentration, but no difference was found in PR mRNA concentration. Recently we have further studied the effect of the presence of the embryo on

sex steroid receptor expression by different methodologies (Thatcher *et al.*, 2003). Endometrial ER mRNA measured both by northern blot and solution hybridization analysis was higher in cyclic cows at day 17. This finding was substantiated by the increase in ER protein measured by immunohistochemistry and western blot analyses (Thatcher *et al.*, 2003). Although there was an increased staining intensity of ER protein in luminal epithelium, this cell type was almost devoid of PR staining. At the same time, PR expression in the uterine glands was higher in pregnant cows. The physiological status did not affect PR mRNA concentration, suggesting the embryo may alter PR expression by post-transcriptional pathways.

In summary, as demonstrated for sheep (Spencer and Bazer, 1995), pregnancy affects the expression of ER in cattle and the decrease observed around the time of maternal recognition of pregnancy suggests this pathway is implicated in the inhibition of luteolysis. Although the lack of PR presence in the luminal epithelium is puzzling, the increase of PR staining found in the uterine glands could explain progesterone stimulus to embryo growth.

## ACKNOWLEDGEMENTS

The present study received financial support from the International Foundation for Science to A.M. (Grant IFS B/3025-2).

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