CSIRO PUBLISHING

Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD11160

The inhibitory effect of progesterone on lactogenesis during pregnancy is already evident by mid- to late gestation in rodents

Constanza M. López-Fontana[A], *María E. Maselli*[A], *Ana M. Salicioni*[B] *and Rubén W. Carón*[A,C]

[A]Instituto de Medicina y Biología Experimental de Cuyo (IMBECU), CONICET, CCT-Mendoza, Argentina.
[B]Department of Veterinary and Animal Sciences, University of Massachusetts-Amherst, Amherst, MA 01003, USA.
[C]Corresponding author. Email: rcaron@mendoza-conicet.gob.ar

Abstract. Lactogenesis is a very complex process highly dependent on hormonal regulation. In the present study the time-course of the inhibitory actions of progesterone on prolactin secretion, mammary gland morphology and lactogenesis from mid- to late gestation in rodents was investigated. Groups of pregnant rats were luteectomised or administered with mifepristone on Day 10, 13, 15 or 17 of gestation and decapitated 28 or 48 h later. Whole-blood samples and the inguinal mammary glands were taken for determinations of hormone levels and for measurement of mammary content of casein and lactose and for tissue morphology analyses, respectively. Luteectomy or mifepristone evoked prolactin increases only after Day 17 of gestation. Mammary content of casein was increased by both treatments regardless of timing or duration. Mifepristone was less effective than luteectomy in inducing lactose production and the effect was only observed after Day 15 of gestation. Analysis of mammary gland morphology confirmed the observed effect of progesterone on lactogenesis. Both treatments triggered remarkable secretory activity in the mammary gland, even without a parallel epithelial proliferation, demonstrating that the mammary epithelium is able to synthesise milk compounds long before its full lobulo–alveolar development is achieved, provided that progesterone action is abolished. Thus, the present study demonstrates that progesterone is a potent hormonal switch for the prolactin and prolactin-like effects on mammary gland development and its milk-synthesising capacity during pregnancy, and that its inhibitory action is already evident by mid-pregnancy in rodents.

Additional keywords: corpus luteum, lactation, mammary gland.

Received 17 June 2011, accepted 18 October 2011, published online 5 December 2011

Introduction

Lactogenesis, defined as the synthesis of milk compounds that takes place at the end of pregnancy, is highly dependent on hormonal regulation. Most mammary gland development occurs during puberty and pregnancy. Puberty is controlled by hormonal signals elicited by the hypothalamic–pituitary–ovarian axis. Ovarian steroids are necessary for ductal morphogenesis of the mammary tissue (for review see Oakes *et al.* 2008). During pregnancy, increased progesterone (P_4) and prolactin (PRL) secretions result in development of the milk-secreting lobulo–alveoli. The effects of PRL on mammary gland development in rodents are both indirect, via modulation of the systemic hormone environment, and direct, via binding to PRL receptors (PRLR) in the mammary epithelium (Brisken *et al.* 1999). Alveolar morphogenesis is initiated in rats by coitus, providing nervous stimulation of pituitary PRL secretion that also sustains ovarian P_4 secretion for up to 10 days, regardless of embryo implantation (Terkel *et al.* 1990). Both hormones induce rapid proliferation of cells in the ductal branches, increasing the epithelial cell number. During the second half of pregnancy, cells of the alveoli differentiate to form the secretory alveolar epithelium, capable of milk production and secretion during lactation. The main hormonal influence during this period is placental lactogen (PL), acting in a PRL-like manner (Deis *et al.* 1989; Caron *et al.* 1994*a*).

Interestingly, P_4 has also been shown to have an inhibitory effect on lactogenesis *in vivo* (Kuhn 1969; Vermouth and Deis 1974; Vermouth and Deis 1975; Bussmann and Deis 1979; Deis *et al.* 1989; Jahn and Deis 1991) and *in vitro* (Rosen *et al.* 1978; Ganguly *et al.* 1982; Levay-Young *et al.* 1990). Studies *in vivo* have clearly shown the inhibitory effect of P_4 on both PRL secretion and lactogenesis at the end of pregnancy (Vermouth and Deis 1974; Caron *et al.* 1994*a*). The induction of lactogenesis in rats by luteolytic agents such as prostaglandin F2α at

the end of pregnancy is prevented by exogenous P_4 (Vermouth and Deis 1975; Bussmann and Deis 1979). Moreover, P_4 antagonists such as mifepristone (MIF) are able to increase mammary contents of casein (CAS) and lactose (LAC) in nineteen day-pregnant rats (Deis *et al.* 1989) to the same extent as the effect seen with prostaglandin F2α (Jahn and Deis 1991).

P_4 withdrawal in both humans and mice initiates mammary secretion, characterised by the accumulation of milk components in the alveoli. Thus, lactogenesis has been most frequently evaluated by the determination of mammary levels of β-CAS and LAC (Deis *et al.* 1989; Jahn and Deis 1991; Caron *et al.* 1994*a*). Even when the effects and mechanisms of progestins on the inhibition of PRL secretion and lactogenesis have been studied, the timing for the influence of P_4 withdrawal during pregnancy is still uncertain. In the present study we aimed to investigate the time-course for the inhibitory actions of P_4 on PRL secretion, on mammary gland morphology and on lactogenesis from mid- to late gestation in rats.

Materials and methods

Animals

Virgin female rats, (3–4 months old, 200–220 g) of the Wistar strain and bred in our animal facility were used. The animals were kept in a light- (lights on 0600–2000 hours) and temperature-controlled (22–24°C) room; rat chow (Cargill, Córdoba, Argentina) and tap water were available *ad libitum*. Vaginal smears were taken daily. Animals were caged individually with fertile males on the night of pro-oestrous, and the presence of spermatozoa was checked in the vaginal smear the following morning. This day was designated as Day 0 of pregnancy. In our animal facility, the majority of animals give birth in the night between Days 21 and 22, although ~25% of the deliveries occur in the afternoon of Day 21.

Animal care and handling were performed according to the NIH guide for the Care and Use of Laboratory Animals (NIH publication no. 86–23, revised 1985 and 1991) and the UK requirements for ethics of animal experimentation (Animals Scientific Procedures, Act 1986). All the experimental procedures were approved by the Institutional Animal Ethics Committee of IMBECU (Instituto de Medicina y Biología Experimental de Cuyo, Mendoza, Argentina).

Experimental design

In order to test the negative regulation exerted by P_4 on mammary gland development and its lactogenic activity during pregnancy, two different experimental approaches were followed: (1) serum P_4 withdrawal by surgical removal of corpora lutea (CLX) or (2) pharmacological inhibition of P_4 binding to its receptor by treatment with MIF.

Groups of pregnant rats had their corpora lutea removed at 0800 hours on Day 10, 13, 15 or 17 of gestation. Those days were chosen according to previous studies showing different regulation of PRL secretion by P_4 on Day 10 of gestation compared with late pregnancy (Jahn *et al.* 1986; Soaje and Deis 1997). CLX was performed under ether anaesthesia by a unique medial incision of the dorsal skin and lateral incisions of the deeper planes at both sides of the spine at the last rim level. For removal of the corpora lutea, the bursa was dissected and all the corpora lutea were extracted using a small straight clamp. Sham-operations were carried out in a similar manner, performing the same incisions of skin and muscle tissue as in CLX animals. Both ovaries were exposed and the bursas were notched without removal of the corpora lutea.

Mifepristone (RU-38486; 17β-hydroxy-11β-(6dimethyl-amino-phenyl) 17α-(prop-1-ynyl) oestra-4,9-dien-3-one) was dissolved in sunflower seed oil (at 2 or 10 $mg\,mL^{-1}$) and injected subcutaneously (2 or 10 $mg\,kg^{-1}$) at 0800 hours on Day 10, 13, 15 or 17 of gestation. The respective control animals were injected subcutaneously with an equivalent volume of vehicle. We compared the two doses of MIF to assess for changes in the effectiveness of the inhibitory action of P_4 on PRL secretion related to the progression of gestation. Both doses of MIF have been previously used to induce PRL secretion under different physiological conditions in rats (Salicioni *et al.* 1993; Caron *et al.* 1994*b*; Soaje and Deis 1997).

The groups were divided in two subgroups and were sacrificed by decapitation either 28 h (at 1200 hours the day following treatment) or 48 h after treatment (at 0800 hours two days after treatment). Trunk whole-blood samples and the inguinal mammary glands were collected and saved for further analysis, i.e. serum hormone levels, mammary content of CAS and LAC and for mammary gland histology. After removal, mammary tissue was stored at −70°C and a small piece was fixed in 10% neutral formalin until processed for the histological study. Blood samples were allowed to clot at room temperature, serum separated and stored at −20°C until assayed for PRL and P_4.

Fetus viability was assessed by examination of all pregnant rats immediately after decapitation. The number and aspect of the feto–placental units were registered and those units with clear signs of fetal resorption were counted.

Serum PRL and P_4 levels measured by radioimmunoassay

PRL was measured by a double-antibody radioimmunoassay (RIA) as previously described (Caron *et al.* 1994*b*), using materials kindly provided by Dr A. F. Parlow and the National Hormone and Pituitary Program (NHPP; Harbor-UCLA Medical Center, Torrance, CA, USA). PRL was radioiodinated using the chloramine-T method. Results are expressed in terms of the rat PRL RP-3 standard preparation of the NHPP. Assay sensitivity was 1 $\mu g\,L^{-1}$ serum and inter and intraassay coefficients of variation were 8 and 3%, respectively. Serum P_4 was measured using a RIA developed in our laboratory (Bussmann and Deis 1979) with antiserum raised in rabbits against progesterone-11β–bovine serum albumin conjugate. Assay sensitivity was less than 1.6 $nmol\,L^{-1}$ serum, and inter and intraassay coefficients of variation were less than 10%.

Mammary gland lactogenic activity by CAS and LAC content, and oxytocin test

Mammary content of CAS and LAC were measured as previously described (Deis *et al.* 1989; Caron *et al.* 1994*a*). Briefly, 200 mg of mammary tissue was cut into small pieces and homogenised in 2 mL 50 mM sodium phosphate buffer, 150 mM

NaCl, 0.1% NaN_3, 0.1% Triton X-100, pH 7.6 with an Ultraturrax homogeniser. After centrifugation at 600*g* for 30 min at 4°C, supernatants were saved and β-CAS content was determined by a homologous radioimmunoassay as previously described (Bussmann and Deis 1984). The standard curve of rat β-CAS was between 0.25 and 512 ng mL^{-1}, and content was expressed per mg of tissue. LAC content in the mammary glands was assessed by the method of Kuhn and Lowenstein (1967), calculated in nmol and normalised per mg of mammary tissue. Lactogenesis was also determined by a modified oxytocin test (Caron *et al.* 1994*a*) consisting of placing a small portion of fresh mammary tissue in a tube containing 100 μU oxytocin (generously provided by Sandoz, Buenos Aires, Argentina) in 0.5 mL saline. If lactogenesis has taken place, the contraction of the mammary tissue produced by oxytocin will expel the accumulated milk to the surrounding liquid, producing a white opalescence. This is a very sensitive qualitative method to determine lactogenesis (Deis 1968).

Mammary gland histology

Two small pieces of inguinal mammary gland from each rat were submerged in 10% buffered neutral formalin (Merck, Buenos Aires, Argentina) during 72 h for fixation. Dehydration was performed by passing through ethanol solutions of increasing graduation and xylol. Paraffin sections were cut at 5 μm thick and hematoxilin and eosin (H&E) were used for staining. Microscopic analysis of the stained preparations was carried out under a Nikon Eclipse E200 Microscope (Nikon Corp., Tokyo, Japan) at a magnification of 100× and digital photographs were taken with a digital still camera Coolpix S10 (Nikon). Mammary gland development after the different treatments was evaluated by measuring the area occupied by the alveoli in 4–8 microscopic fields per section and two sections per animal (six rats per group) using the ImageJ 1.42q software available at the NIH site (http://rsb.info.nih.gov/ij, accessed 12 April 2011). Each area was expressed as a percentage of the whole field.

Statistics

Values are given as means ± s.e.m. of six to nine animals per group, unless indicated otherwise. All statistical analyses were performed using GraphPad Prism 5.01 software (GraphPad Software Inc., san, CA, USA). Two-way analysis of variance (ANOVA II) was used for comparing the effects between each treatment and their respective control group over time of pregnancy. Post hoc comparisons between means were made by Bonferroni's test. The effects of the different doses of MIF were compared using ANOVA II and Bonferroni's test as post hoc. The effect of time on the response produced by each treatment was individually analysed using ANOVA I and Bonferroni's test as post hoc. When variances were not homogeneous logarithmic transformation of data was applied. For the analysis of oxytocin test results, fractions of positive tests were compared by chi-square test for trend. The percentages of resorbed fetuses were compared by Fisher's exact test. Differences were considered to be significant for $P < 0.05$.

Table 1. Percentage of fetal resorptions in CLX or MIF-treated pregnant rats on different days of gestation

Rats were sacrificed 48 h after CLX surgery or MIF (2 mg kg^{-1}) administration. All fetuses with clear signs of resorption were counted and expressed as percentage of total fetuses. Statistical analysis was performed by Fisher's exact test

Treatment day	CLX (%)	MIF (%)	*P* value
Day 10	69	58.3	0.14
Day 13	14.8	8.1	0.18
Day 15	10.0	9	1.0
Day 17	50	47.7	0.89

Results

Fetal viability and resorption

In control animals, pregnancy was not affected by the simulated surgery or vehicle administration ($P > 0.05$). Both CLX and MIF (2 mg kg^{-1}) administration affected pregnancy at the same extent 48 h after treatment. The number of feto–placental units (CLX, 10.2 ± 0.4 total fetuses vs MIF, 9.7 ± 0.4 total fetuses) and the percentage of fetal resorptions (Table 1) were found to be not statistically different between both treatments. These results suggest that our antiprogesterone treatments have similar impacts at the uterine level, the main target of P_4 action in pregnant rats. Furthermore, pregnancy was interrupted in all animals 48 h after 10 mg kg^{-1} MIF irrespective of the day of administration.

Time-dependent PRL response to P_4 inhibition during pregnancy

First we aimed to determine whether serum PRL levels were dependent on P_4 during early pregnancy in comparison to late phases. CLX is one of the most effective methods of removing the main source of circulating P_4 whilst leaving other ovarian functions intact, i.e. oestrogen secretion (Bussmann *et al.* 1983). MIF treatment has also proven to be a useful tool to block the action of P_4 on its targets (Bussmann *et al.* 1983; Stocco *et al.* 2001; Telleria *et al.* 2001; Soaje *et al.* 2006). Thus, comparing the effects of both treatments is useful for assessment of the intrinsic effects of P_4. As shown in Fig. 1*a*, serum PRL levels, measured 28 h after CLX performed on Day 10, 13 or 15 of gestation, were similar to those found in sham-operated animals ($P > 0.05$); the difference between the two groups became significant when CLX was performed on 17 day-pregnant rats ($P < 0.001$, Fig. 1*a*). PRL levels were similar between CLX and control animals if measured 48 h after operating at all time points (data not shown).

PRL response to the administration of the P_4 antagonist MIF appeared 48 h after injection. Thus, we analysed PRL levels in experimental and control rats, either 28 h after CLX or 48 h after MIF administration, when both treatments had been shown to be effective in increasing PRL secretion. As shown in Fig. 1*b*, low doses (2 mg kg^{-1}) of MIF were not effective on PRL release until late in pregnancy ($P < 0.001$ when administered on Day 17). In comparison, 13- or 15-day pregnant rats were already responsive to 10 mg kg^{-1} of the P_4 inhibitor and showed

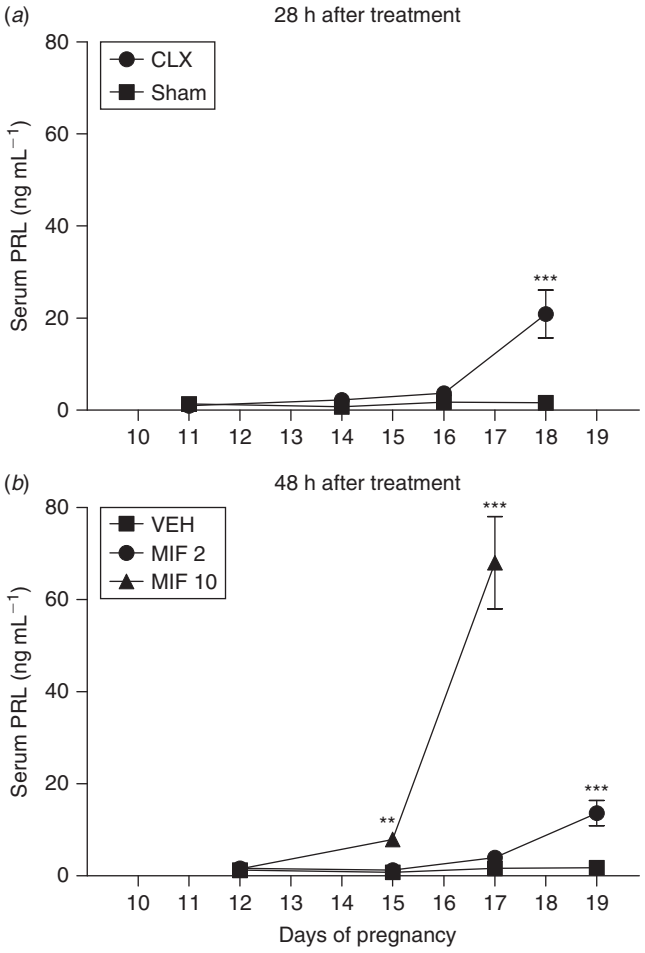

Fig. 1. Serum PRL levels (*a*) 28 h after CLX or (*b*) 48 h after administration of 2 or 10 mg kg^{-1} MIF to pregnant rats on Day 10, 13, 15 or 17 of gestation. Since results 48 h after CLX and 28 h after MIF were not significantly different between experimental and control rats, they are not shown in the figure. Control groups are sham-operated pregnant rats and vehicle-administered pregnant rats, respectively. Each time point represents when animals were decapitated to take samples. Each value is mean ± s.e.m. of six to nine rats. ** $P < 0.01$, *** $P < 0.001$ compared with control. Two-way analysis of variance (ANOVA II) and Bonferroni's test were used to compare the effects between each treatment to their respective control group over time of pregnancy.

a significant increase in serum PRL 48 h later ($P < 0.01$ and $P < 0.001$, respectively). Administration of MIF in low doses (2 mg kg^{-1}) to 10-, 13-, 15- or 17-day pregnant rats did not significantly modify serum PRL measured 28 h later (data not shown), further indicating that the PRL response to treatment with the P_4 antagonist was delayed and only detectable when evaluated two days after injection.

CLX decreased serum P_4 concentration to similar values on different days of gestation during gestation ($P < 0.001$, Table 2). This clear drop in serum P_4 after CLX has been described previously in pregnant rats (Bussmann *et al.* 1983). Interestingly, treatment with 2 mg kg^{-1} MIF also induced a significant fall in the circulating levels of P_4 only on Day 10 ($P < 0.05$) while 10 mg kg^{-1} MIF decreased P_4 levels at all time points studied ($P < 0.01$, Table 2). This dose-dependent effect suggests an auto-regulatory, negative feedback mechanism in agreement with previous studies (Kawano *et al.* 1988; Telleria and Deis 1994; Telleria *et al.* 1995).

Mammary gland lactogenic activity in response to P_4 inhibition during pregnancy

Next we assessed mammary gland lactogenic activity in response to P_4 inhibition on different days of pregnancy according to three parameters: quantitative changes in mammary content of CAS, content of LAC and a qualitative change by presence of milk in mammary tissue. Fig. 2*a* shows the effect of CLX on the content of mammary CAS determined 48 h after treatment. Mammary gland response to P_4 withdrawal was already evident by Day 12 of pregnancy, and stayed significantly higher than in sham-operated rats at all time points analysed ($P < 0.001$, Fig. 2*a*). A similar response was also observed 28 h after CLX (data not shown). A comparable, yet more gradual, response was elicited in pregnant animals treated with 2 mg kg^{-1} MIF, where the content of mammary CAS increased significantly 48 h after treatment on Day 10 ($P < 0.01$), 13 ($P < 0.05$) and 15 ($P < 0.001$), and reached a maximum response on Day 17 respective to controls ($P < 0.001$, Fig. 2*c*). Moreover, the injection of 10 mg kg^{-1} promoted a significantly greater response when compared with the lower dose ($P < 0.001$ on Day 13 and $P < 0.05$ on Day 15 of treatment). Furthermore, this higher dose evoked a more significant increase in mammary CAS respective to the controls from Day 10 of treatment on ($P < 0.001$, Fig. 2*c*).

Table 2. Effect of CLX or treatment with MIF on serum levels of progesterone on different days of gestation

Sampling was performed 48 h after treatment (surgery or administration of vehicle or MIF). Values correspond to the mean ± s.e.m. Number of animals per group is shown in brackets. * $P < 0.05$ compared with vehicle-treated animals; ** $P < 0.01$ compared with vehicle or 2 mg kg^{-1} MIF administration; *** $P < 0.001$ compared with sham-operated animals. [a]$P < 0.01$ with respect to other days of pregnancy. Two-way analysis of variance (ANOVA II) and Bonferroni's test were used to compare the effects between each treatment to their respective control group over time of pregnancy

Treatment day	Serum progesterone (ng mL^{-1})				
	SHAM	CLX	Vehicle	MIF 2 mg kg^{-1}	MIF 10 mg kg^{-1}
Day 10	47.0 ± 8.6 (5)	7.8 ± 1.8 (7)***	55.0 ± 4.2 (5)	24.2 ± 6.2 (7)*[a]	7.7 ± 1.0 (4)**[a]
Day 13	56.9 ± 13.4 (5)	3.0 ± 0.6 (7)***	71.0 ± 3.8 (5)	65.1 ± 2.9 (10)	47.8 ± 4.5 (12)**
Day 15	83.0 ± 8.7 (5)	1.5 ± 0.3 (7)***	65.1 ± 2.3 (5)	62.1 ± 3.7 (5)	37.4 ± 4.1 (6)**
Day 17	75.5 ± 7.0 (5)	3.1 ± 1.3 (7)***	52.7 ± 2.7 (4)	39.4 ± 5.5 (11)	–

In comparison to CAS, a less pronounced and delayed response of the synthesis of LAC to the lack of P_4 action was observed (Fig. 2*b*). Removal of corpora lutea on Day 10 of gestation did not modify the amount of LAC in mammary gland measured 48 h after operation. This response became significant after CLX on Day 13, 15 or 17 of gestation ($P < 0.001$). A maximum increase in mammary LAC induced by CLX was observed at 0800 hours on Day 17 of gestation. As seen in Fig. 2*d*, 2 mg kg^{-1} MIF caused a significant increase in mammary LAC 48 h later in animals treated on Day 17 ($P < 0.01$). As predicted, the higher dose of MIF elicited an earlier response with an increase in LAC content already detectable in 15-day pregnant animals ($P < 0.01$).

Results obtained by the oxytocin test (Table 3), which detects the macroscopic presence of milk, confirmed our findings based on mammary content of CAS and LAC. Bilateral CLX early in pregnancy (Day 10) induced a positive oxytocin test in at least 50% of the animals tested when determined 28 h after treatment. All the animals showed positive tests when corpora lutea were removed later during pregnancy. In the groups injected with

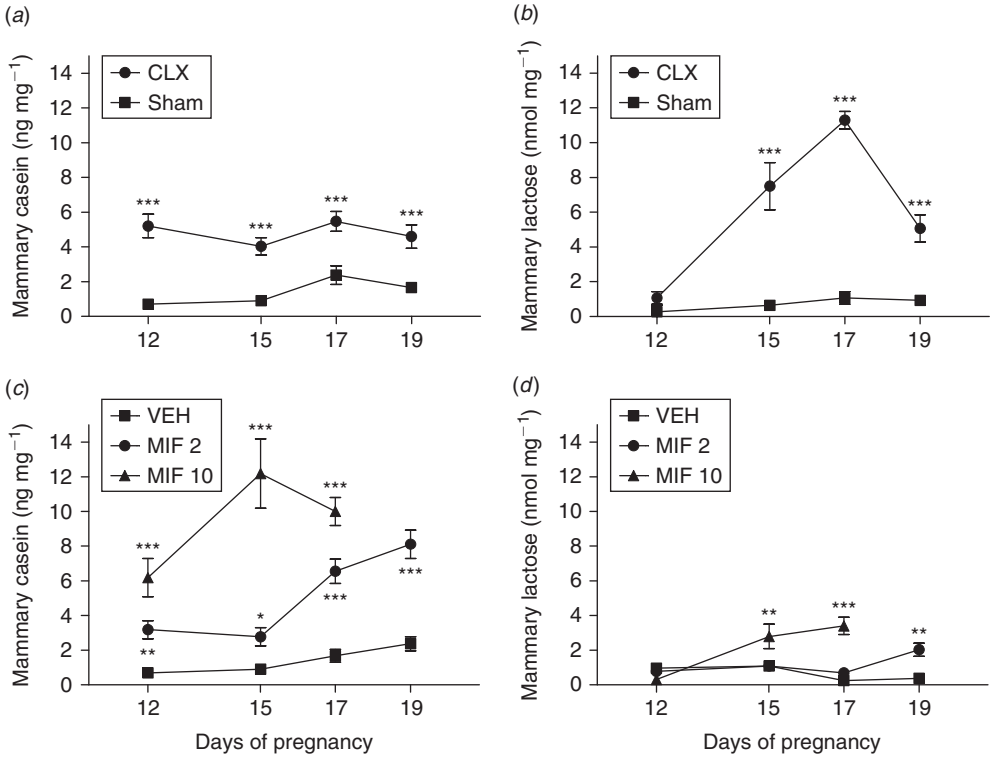

Fig. 2. Mammary contents of (*a*, *c*) casein or (*b*, *d*) lactose (*a*, *b*) 48 h after CLX or (*c*, *d*) administration of 2 or 10 mg kg^{-1} MIF to pregnant rats on Day 10, 13, 15 or 17 of gestation. Control groups are: (*a*, *b*) sham-operated pregnant rats and (*c*, *d*) vehicle-administered pregnant rats. Each time point represents when animals were decapitated to take samples. Each value is mean ± s.e.m. of six to nine rats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control. Two-way analysis of variance (ANOVA II) and Bonferroni's test were used to compare the effects between each treatment to their respective control group over time of pregnancy.

Table 3. Determination of lactogenesis according to the oxytocin test

T+, number of animals with positive test; TT, total number of tested animals per group. Fractions were compared by chi-square test for trend. Comparisons were made between each treatment and the control group and between the two antiprogesterone treatments at 28 and 48 h. All groups were statistically different from the control group ($P < 0.001$) except for MIF on Day 10. Superscript letter indicate significant difference between treatments at [a]28 h and [b]48 h ($P < 0.001$). Chi-square test and Fisher exact test were used for comparisons

Treatment day	Control	CLX (T+/TT)		MIF (T+/TT)	
		28 h	48 h	28 h	48 h
Day 10	0/12	6/11[a]	5/19[b]	0/8[a]	0/7[b]
Day 13	0/11	10/10[a]	8/8[b]	2/9[a]	1/10[b]
Day 15	0/13	7/7[a]	13/13[b]	5/8[a]	6/10[b]
Day 17	0/11	7/7	7/7	7/8	14/17

2 mg kg^{-1} MIF, the positive response to the oxytocin test was delayed and less significant when compared with CLX animals ($P < 0.001$). No statistically significant differences were found comparing 28 and 48 h in each treatment.

Effect of P_4 inhibition on mammary gland tissue differentiation during pregnancy

The effect of P_4 on the differentiation of the mammary gland throughout pregnancy was further evaluated by histological observation and measurement of the area occupied by alveolar structures as previously described (Wlodek *et al.* 2009). Fig. 3 shows photomicrographs of H&E-stained mammary tissue from 12-, 15-, 17- or 19-day pregnant rats. Discrete lobe–alveolar development of the mammary gland was observed, without signs of secretion, in 12- and 15-day pregnant animals after sham-operation (Fig. 3*a*, *c*), while alveolar development was more evident in CLX animals ($P < 0.001$, Fig. 3*b*, *d*, *i*). In the last third of gestation, mammary tissue from sham-operated animals showed some evidence of lobe–alveolar differentiation and no secretory activity (Fig. 3*e*). In contrast, mammary tissue from animals CLX on Day 15 of pregnancy and sacrificed 48 h later (Fig. 3*f*), displayed much greater alveolar development ($P < 0.001$ in Fig. 3*i*) and clear signs of secretion. On Day 19 of gestation, 48 h after sham operation or CLX (Fig. 3*g*, *h*), there was a profuse lobular development arranged in closely packed and expanded alveoli showing abundant secretion. This development was more abundant after CLX than in sham-operated rats ($P < 0.05$).

Mammary gland response to pharmacological inhibition of P_4 action by treatment with MIF is shown in Fig. 4; quantifications of the relative percentages of the alveolar areas are shown in Fig. 4*m*. Important increases of these areas ($P < 0.001$) and signs of secretion could be observed 48 h after the administration of 2 mg kg^{-1} of the antagonist from Day 13 of pregnancy onwards compared with the control groups (Fig. 4*e*, *h*, *k*). Furthermore, the injection of 10 mg kg^{-1} of the P_4 inhibitor induced significant augmentation in the areas occupied by alveoli ($P < 0.001$) with evident secretion in the alveolar lumen, and decreased the relative number of adipose cells (Fig. 4*f*, *i*, *l*). Our results indicate that 2 and 10 mg kg^{-1} MIF elicited a similar increase in the alveolar area ($P > 0.05$, Fig. 4*m*).

Discussion

In female rats, P_4 has been shown to exert a dual regulatory effect on serum PRL levels (Jahn *et al.* 1986). During the first days of gestation, P_4 stimulates PRL secretion, whereas from Day 11 of pregnancy, the waves of hypophysial PRL are negatively regulated by the tonic secretion of PL associated with high levels of circulating P_4. The evidence presented here clearly shows that, during gestation, the response of the hypothalamus–pituitary–axis to the drop of circulating P_4 occurs in a gradual manner, with a moderate increase in PRL secretion from Day 15. CLX performed on Day 17 induces a noteworthy increase of serum PRL followed by a decrease to basal levels one day later (20.9 ± 5.2 vs 2.1 ± 0.5 ng mL^{-1}), corresponding to a phased PRL response, also described by others (Jahn *et al.* 1986). This response clearly indicates that, at mid-gestation, the inhibition of PRL secretion depends on the action of P_4, probably associated with the effect of PL. Accordingly, Wlodek *et al.* (2009) have shown that uteroplacental insufficiency is able to induce early lactogenesis at the end of pregnancy due to a drop in circulating P_4 and probably in PL, associated with increased secretion of PRL. Interestingly, lactogenesis, evaluated as the increase of the expression of α-lactoalbumin, β-CAS and whey acidic protein, implicated as markers of mammary epithelial cell differentiation and the onset of secretory activation, was partially reverted by administration of P_4 to the dams (Wlodek *et al.* 2009).

In the present study the lowest dose of MIF did not modify the secretion of PRL up to Day 17 of gestation. Two days later, the same treatment triggered an increase in PRL comparable to that induced by removal of corpora lutea, suggesting a progressive change in the responsiveness of PRL secretion to the inhibitory actions of P_4 throughout pregnancy. Furthermore, treatment of pregnant animals on Day 15 with a higher dose of MIF evoked PRL secretion to values higher than with CLX, suggesting a dose-dependent action of MIF on PRL secretion. In fact, low doses of MIF are not sufficiently potent to induce PRL secretion, but can enhance the effect of other stimuli, such as suckling (Soaje *et al.* 2002), whereas high doses are effective by themselves. In accordance with studies by Vermouth and Deis (1975), and taking into consideration that MIF can also bind hypothalamic receptors of P_4 (Shi and Zhu 1990), our results provide evidence of the inhibitory role of P_4 on PRL secretion throughout pregnancy, probably acting on the hypothalamus. Grattan and Averill (1990) have shown that a spontaneous peak of PRL occurs at the end of pregnancy in rats, and that this augmented secretion is linked to the daily photoperiod and is characterised by a nocturnal surge in the dark period preceding parturition. This surge is inhibited by P_4 or ovariectomy, and it can be advanced 24 h by oestradiol treatment in the absence of the ovaries. This is probably the case in our CLX or MIF-treated rats since they have no action of P_4 but still retain circulating oestradiol. It has been clearly demonstrated that the decrease in dopamine is dissociated from the antepartum surge of PRL and that dopamine is not the only regulator of PRL secretion at this time (Grattan and Averill 1992). A loss in the responsiveness to PRL of the dopaminergic autoregulatory short loop has been pointed out as an important physiological mechanism to allow for the hypersecretion of PRL during lactation (Grattan and Averill 1995).

The interplay between P_4 and PRL in the development of the mammary gland was also evaluated throughout pregnancy. During gestation the mammary epithelium undergoes a notable lobulo–alveolar development that allows synthesis of all components of milk later on, during lactation. The transitional period from growth to differentiation is a complex mechanism regulated by several hormones (Neville *et al.* 2002; Anderson *et al.* 2007; Russo and Russo 2008). The synthesis of milk components such as CAS and LAC is mainly induced by PRL and modulated by glucocorticoids and P_4, the former being stimulatory and the latter being inhibitory. Several studies have demonstrated the presence of P_4 and glucocorticoid receptors in mammary tissue from pregnant animals (Haslam and Shyamala 1980, 1981; Quirk *et al.* 1982). Glucocorticoids

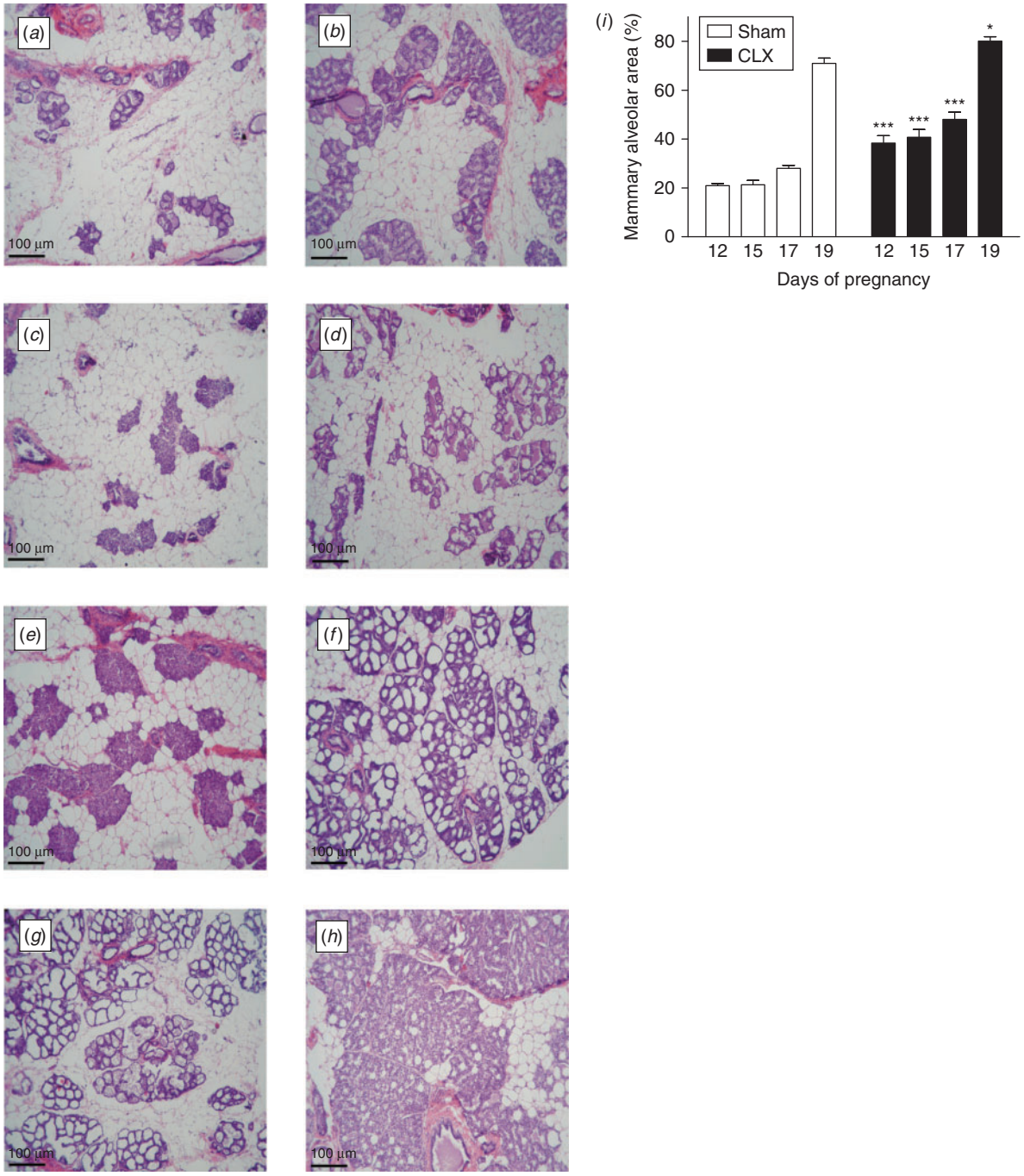

Fig. 3. Representative photomicrographs (100×) of H&E-stained mammary tissue from (*a, b*) 12-, (*c, d*) 15-, (*e, f*) 17- and (*g, h*) 19-day pregnant rats, 48 h after CLX (right panels) or sham operation (left panels). Mammary tissue from sham-operated rats can be seen with a discrete lobe–alveolar development and without any evidence of secretion (*a*, *c*, *e*). In the last third of gestation, sham-operated animals show a slight lobe–alveolar differentiation and no secretory activity (*g*). In contrast, CLX produced more evident alveolar development and the ducts showed signs of abundant epithelial secretory activity (*b*, *d*, *f*). On Day 19 of gestation, 48 h after CLX, there was greater alveolar development and clear signs of secretion (*h*). (*i*) Alveolar development of the mammary glands at different days of pregnancy after CLX (black bars) or sham operation (open bars). Quantification was performed by measuring the area occupied by the epithelium in 4–8 fields (100×) of each preparation. Each area was expressed as a percentage of the whole field. * $P < 0.05$, *** $P < 0.001$ compared with control. Two-way analysis of variance (ANOVA II) and Bonferroni's test were used to compare the effects between each treatment to their respective control group over time of pregnancy.

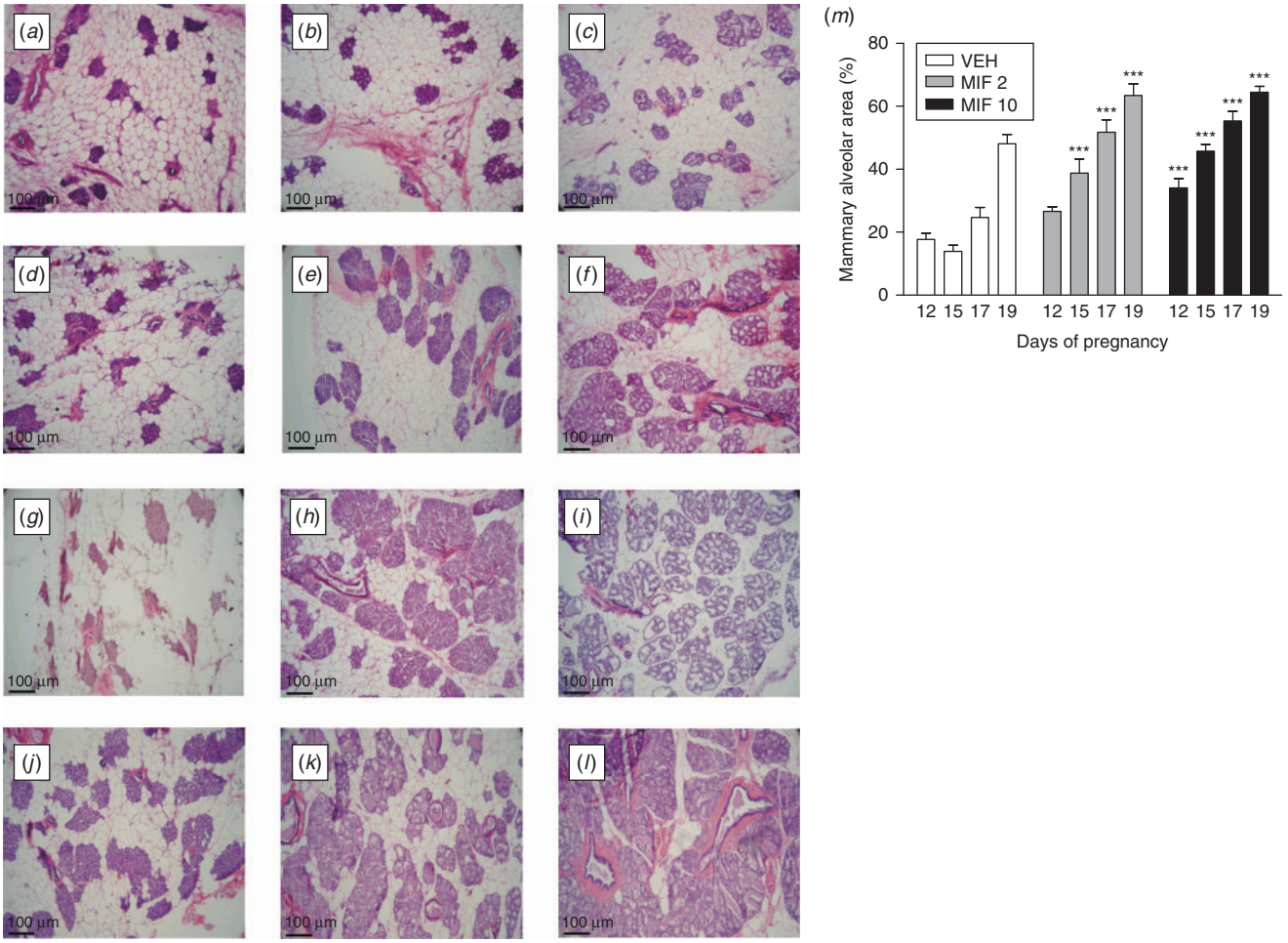

Fig. 4. Representative photomicrographs (100×) of H&E-stained mammary tissue from (*a*, *b*, *c*) 12-, (*d*, *e*, *f*) 15-, (*g*, *h*, *i*) 17- or (*j*, *k*, *l*) 19-day pregnant rats, 48 h after vehicle (left panels), 2 mg kg^{-1} MIF (middle panels) or 10 mg kg^{-1} MIF (right panels). Mammary tissue of animals treated with vehicle shows poorly developed epithelium and abundant stromal elements (*a*, *d*, *g*). The mammary tissue of rats from 19 days of gestation treated with vehicle 48 h earlier (*j*) shows lobe–alveolar structures essentially with the same characteristics as those described above in animals with simulated operation (Fig. 3*g*). The administration of 2 mg kg^{-1} MIF increased alveolar size and induced secretory activity in the mammary gland of 15-day pregnant animals, apparently without changing the number of glandular acini (*e*). In contrast, when 10 mg kg^{-1} of the progesterone antagonist was used, dilation and alveolar mammary secretion was more noticeable with remarkable changes in the alveoli and a decrease in the relative number of adipose cells and vacuolisation from Day 12 on (*c*, *f*). Mammary tissue, obtained 48 h after treatment with 10 mg kg^{-1} MIF (*i*), shows no major changes compared with the lowest dose of antagonist (*h*). In both cases, the increase in the lobular size with bigger cells and many vacuoles is clear. The alveolar lumen is evident and the number of adipocytes is limited. On Day 19, 48 h after treatment with 2 mg kg^{-1} MIF (*k*), alveolar arrangement is even more evident, cytoplasm volume has increased significantly and the profuse vacuolisation gives a spongy aspect to the mammary tissue. Secretion is evident in the alveolar lumen. A very similar picture is shown 48 h after 10 mg kg^{-1} MIF on Day 17 of gestation (*l*). (*m*) Alveolar development of the mammary glands at different days of pregnancy after 2 mg kg^{-1} MIF (grey bars), 10 mg kg^{-1} MIF (black bars) or vehicle administration (open bars). Quantification was performed by measuring the area occupied by the epithelium in 4–8 fields (100×) of each preparation. Each area was expressed as a percentage of the whole field. *** $P < 0.001$ compared with control. Two-way analysis of variance (ANOVA II) and Bonferroni's test were used to compare the effects between each treatment to their respective control group over time of pregnancy.

stimulate the synthesis of CAS and α-lactoalbumin *in vitro* (Quirk *et al.* 1986), and P_4 is a potent inhibitor of milk synthesis during gestation, even though its presence is important for lobulo-alveolar development (Topper and Freeman 1980). Early studies have also demonstrated a clear correlation between lower serum P_4 and a simultaneous release of PRL as a determinant step in the initiation of lactogenesis in the rat at the end of pregnancy (Bussmann and Deis 1979). Since corticoids and P_4 have opposite effects on lactogenesis, we can assume that the increase induced in lactogenesis markers by MIF is due to the antiprogesterone effect and not to an anti-glucocorticoid action, since that would produce an inhibition of lactogenesis rather than an increase.

Our present study provides further evidence of the importance of P_4 in the synthesis of CAS during pregnancy. The increase of the mammary content of CAS induced by P_4 withdrawal in the absence of PRL secretion suggests that PL is able to substitute PRL functions at the peripheral level

promoting lactogenesis, further supporting previous studies (Bussmann and Deis 1985). However, another possibility includes an increase in local synthesis of PRL in the mammary gland (Lkhider *et al.* 1997). To note, the present study clearly shows that in both cases, the lactogenic capability is already evident from Day 10 of pregnancy onwards, as long as P_4 action is abolished.

Contrary to our observations on the steady increase in the synthesis of CAS after CLX, we found that the P_4-dependent synthesis of LAC was progressive and delayed, suggesting a more complex mechanism of hormonal regulation involving PL II, oestrogens, PRL and P_4. Accordingly, type II PL, which is secreted during the second half of pregnancy in rats, is a more potent stimulatory factor on the synthesis of α-lactoalbumin than type I, which is produced during the first half of gestation (Fielder *et al.* 1992). Moreover, the increase in mammary content of LAC on Days 19 and 20 of pregnancy is coincident with the stimulatory action of oestrogen, whose concentration is augmented during this period (Kuhn *et al.* 1980). The association of oestrogens and increased levels of PRL facilitates the synthesis of LAC (Bussmann and Deis 1979; Bussmann *et al.* 1983). In physiological conditions, at the end of pregnancy, the decrease of serum P_4 induced by prostaglandin F2α promotes the increase of PRLR on the mammary epithelium, allowing PRL to display lactogenic actions facilitated by oestrogen on days close to parturition (Bussmann and Deis 1979). According to this complex hormonal regulation, the increase of LAC induced by the antiprogesterone treatments in our rats was delayed with respect to the increase in CAS. Moreover, when we compared both treatments, the increase of LAC induced by MIF was even more delayed and less evident than the one promoted by CLX, reaching significance by Day 17. Even if we cannot rule out that this lower response is due to an antiglucocorticoid effect of MIF, *in vitro* studies have demonstrated that this action is less significant than the antiprogesterone effect (Attardi *et al.* 2004). Moreover, on Days 12 to 14 of gestation, the mammary gland is more sensitive to the negative influence of P_4 than to the stimulatory actions of glucocorticoids (Jahn *et al.* 1987). Since the lowest dose of MIF did not induce PRL secretion during this period of pregnancy, the increase of mammary LAC and CAS should be due to the lactogenic effect of placental factors associated with a weak antiprogesterone effect of MIF at the mammary level. On the contrary, PRL release induced by $10\,mg\,kg^{-1}$ MIF most likely enhanced the effect of PL on the mammary gland, allowing a higher synthesis of CAS from Day 12 of gestation and bringing the mammary increase of LAC forward.

During gestation the mammary gland reaches its maximal histological development under the influence of a delicate and precise balance between pituitary, ovarian, placental and adrenal hormones (Neville *et al.* 2002). In most species, two major pregnancy-related changes in the mammary epithelium have been described: epithelial proliferation is the main change during the first days of pregnancy, while cell differentiation is the main characteristic later on (Forsyth 1994). In rodents, P_4 seems to participate in both processes, probably acting on different types of receptors. P_4 receptor A (PRA) and P_4 receptor B (PRB) expression are temporally and spatially separated during mammary gland development. Only PRA is highly expressed in the immature and adult virgin mammary gland, suggesting a role in early proliferation and juvenile development. In contrast, PRB is seen only during pregnancy, mainly in alveolar epithelial cells. Moreover, colocalisation of PRB and the proliferation marker BrdU and with cyclin D1 strongly suggests that PRB is involved in the rapid proliferation of epithelial cells during pregnancy (Aupperlee *et al.* 2005). Moreover, P_4 and PR have been involved in the inhibition of lactogenesis by decreasing the expression of PRLR and β-CAS (Nishikawa *et al.* 1994). In the present study the analysis of mammary gland morphology confirmed the observed effect of P_4 on lactogenesis as evaluated by the mammary content of LAC and CAS. We found that major changes in the mammary epithelium were already produced two days after antiprogesterone treatments, from Day 10 on, when an increase in both the alveolar proportion and the consequent secretory activity was detected. The decrease in circulating P_4, or the lack of its effect at the receptor level, is able to trigger remarkable secretory activity in the mammary gland and progressive differentiation of the epithelium. It is interesting to note that all these changes in mammary responsiveness to P_4 is paralleled by variations in the central response to the steroid in terms of maternal behaviour (Bridges 1984; Siegel 1986). Thus, two essential actions of P_4 intended to secure the survival of the litter, mammary preparation for lactation and the onset of maternal behaviour of the dam, occur with a similar time pattern, showing a priming effect early in gestation and a dependence on P_4 withdrawal for their full establishment (Rosenblatt *et al.* 1988, 1998).

In summary, in the present study we demonstrate that the mammary gland is able to synthesise milk compounds throughout pregnancy long before its full lobe–alveolar development is achieved, provided that P_4 action is abolished. Furthermore, proliferation of the mammary epithelium beyond Day 10 of gestation contributes to an increase in milk production.

Acknowledgements

The authors wish to thank Dr A. F. Parlow from the National Hormone and Peptide Program, NIADDK, for rat PRL RIA reagents, Mrs. Elina Guiñazú de Di Nasso and Mr. Juan Rosales for their excellent technical assistance and Dr O. Alvarez for advice with statistical analysis. This paper is dedicated to the memory of Dr Ricardo P. Deis, who was a pioneer on PRL and lactation physiology, and whose suggestions and comments greatly enriched this work. This work was supported by grant PICT 99 05–06877 from Agencia Nacional de Promoción Científica y Tecnológica and partially by PIP number 11220090100145 from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina). R. W. C. is a scientist from CONICET.

References

Anderson, S. M., Rudolph, M. C., McManaman, J. L., and Neville, M. C. (2007). Key stages in mammary gland development. Secretory activation in the mammary gland: it's not just about milk protein synthesis! *Breast Cancer Res.* **9**, 204–218. doi:10.1186/BCR1653

Attardi, B. J., Burgenson, J., Hild, S. A., and Reel, J. R. (2004). *In vitro* antiprogestational/antiglucocorticoid activity and progestin and glucocorticoid receptor binding of the putative metabolites and synthetic derivatives of CDB-2914, CDB-4124 and mifepristone. *J. Steroid Biochem. Mol. Biol.* **88**, 277–288. doi:10.1016/J.JSBMB.2003.12.004

Aupperlee, M. D., Smith, K. T., Kariagina, A., and Haslam, S. Z. (2005). Progesterone receptor isoforms A and B: temporal and spatial differences in expression during murine mammary gland development. *Endocrinology* **146**, 3577–3588. doi:10.1210/EN.2005-0346

Bridges, R. S. (1984). A quantitative analysis of the roles of dosage, sequence and duration of oestradiol and progesterone exposure in the regulation of maternal behaviour in the rat. *Endocrinology* **114**, 930–940. doi:10.1210/ENDO-114-3-930

Brisken, C., Kaur, S., Chavarria, T. E., Binart, N., Sutherland, R. L., Weinberg, R. A., Kelly, P. A., and Ormandy, C. J. (1999). Prolactin controls mammary gland development via direct and indirect mechanisms. *Dev. Biol.* **210**, 96–106. doi:10.1006/DBIO.1999.9271

Bussmann, L. E., and Deis, R. P. (1979). Studies concerning the hormonal induction of lactogenesis by prostaglandin F2 alpha in pregnant rats. *J. Steroid Biochem.* **11**, 1485–1489. doi:10.1016/0022-4731(79)90125-0

Bussmann, L. E., and Deis, R. P. (1984). Gamma-glutamyltransferase activity in mammary gland of pregnant rats and its regulation by ovarian hormones, prolactin and placental lactogen. *Biochem. J.* **223**, 275–277.

Bussmann, L. E., and Deis, R. P. (1985). Hormonal regulation of casein synthesis at the end of pregnancy. *Mol. Cell. Endocrinol.* **39**, 115–118. doi:10.1016/0303-7207(85)90127-3

Bussmann, L. E., Koninckx, A., and Deis, R. P. (1983). Effect of oestrogen and placental lactogen on lactogenesis in pregnant rats. *Biol. Reprod.* **29**, 535–541. doi:10.1095/BIOLREPROD29.3.535

Caron, R. W., Jahn, G. A., and Deis, R. P. (1994*a*). Lactogenic actions of different growth hormone preparations in pregnant and lactating rats. *J. Endocrinol.* **142**, 535–545. doi:10.1677/JOE.0.1420535

Caron, R. W., Salicioni, A. M., and Deis, R. P. (1994*b*). Mifepristone treatment demonstrates the participation of adrenal glucocorticoids in the regulation of oestrogen-induced prolactin secretion in ovariectomized rats. *J. Steroid Biochem. Mol. Biol.* **48**, 385–389. doi:10.1016/0960-0760(94)90079-5

Deis, R. P. (1968). Oxytocin test to demonstrate the initiation and end of lactation in rats. *J. Endocrinol.* **40**, 133–134. doi:10.1677/JOE.0.0400133

Deis, R. P., Carrizo, D. G., and Jahn, G. A. (1989). Suckling-induced prolactin release potentiates mifepristone-induced lactogenesis in pregnant rats. *J. Reprod. Fertil.* **87**, 147–153. doi:10.1530/JRF.0.0870147

Fielder, P. J., Thordarson, G., English, A., Rosenfeld, R. G., and Talamantes, F. (1992). Expression of a lactogen-dependent insulin-like growth factor-binding protein in cultured mouse mammary epithelial cells. *Endocrinology* **131**, 261–267. doi:10.1210/EN.131.1.261

Forsyth, I. A. (1994). Comparative aspects of placental lactogens: structure and function. *Exp. Clin. Endocrinol.* **102**, 244–251 [Review] [61 refs]. doi:10.1055/S-0029-1211288

Ganguly, R., Majumder, P. K., Ganguly, N., and Banerjee, M. R. (1982). The mechanism of progesterone–glucocorticoid interaction in regulation of casein gene expression. *J. Biol. Chem.* **257**, 2182–2187.

Grattan, D. R., and Averill, R. L. (1990). Effect of ovarian steroids on a nocturnal surge of prolactin secretion that precedes parturition in the rat. *Endocrinology* **126**, 1199–1205. doi:10.1210/ENDO-126-2-1199

Grattan, D. R., and Averill, R. L. (1992). Neurohormonal factors involved in the control of the nocturnal prolactin surge that precedes parturition in the rat. *J. Neuroendocrinol.* **4**, 167–172. doi:10.1111/J.1365-2826.1992.TB00155.X

Grattan, D. R., and Averill, R. L. (1995). Absence of short-loop autoregulation of prolactin during late pregnancy in the rat. *Brain Res. Bull.* **36**, 413–416. doi:10.1016/0361-9230(94)00216-N

Haslam, S. Z., and Shyamala, G. (1980). Progesterone receptors in normal mammary gland: receptor modulations in relation to differentiation. *J. Cell Biol.* **86**, 730–737. doi:10.1083/JCB.86.3.730

Haslam, S. Z., and Shyamala, G. (1981). Relative distribution of oestrogen and progesterone receptors among the epithelial, adipose and connective tissue components of the normal mammary gland. *Endocrinology* **108**, 825–830. doi:10.1210/ENDO-108-3-825

Jahn, G. A., and Deis, R. P. (1991). Involvement of the adrenergic system on the release of prolactin and lactogenesis at the end of pregnancy in the rat. *J. Endocrinol.* **129**, 343–350. doi:10.1677/JOE.0.1290343

Jahn, G. A., Alonso, N., and Deis, R. P. (1986). Ovarian and feto–placental factors and the regulation of prolactin release during pregnancy in the rat. *J. Reprod. Fertil.* **77**, 125–133. doi:10.1530/JRF.0.0770125

Jahn, G. A., Houdebine, L. M., and Djiane, J. (1987). Antiprogesterone and antiglucocorticoid actions of RU 486 on rabbit mammary gland explant cultures. Evidence for a persistent inhibitory action of residual progesterone upon the mammary tissue. *J. Steroid Biochem.* **28**, 371–377. doi:10.1016/0022-4731(87)91053-3

Kawano, T., Okamura, H., Tajima, C., Fukuma, K., and Katabuchi, H. (1988). Effect of RU 486 on luteal function in the early pregnant rat. *J. Reprod. Fertil.* **83**, 279–285. doi:10.1530/JRF.0.0830279

Kuhn, N. J. (1969). Progesterone withdrawal as the lactogenic trigger in the rat. *J. Endocrinol.* **44**, 39–54. doi:10.1677/JOE.0.0440039

Kuhn, N. J., and Lowenstein, J. M. (1967). Lactogenesis in the rat. Changes in metabolic parameters at parturition. *Biochem. J.* **105**, 995–1002.

Kuhn, N. J., Carrick, D. T., and Wilde, C. J. (1980). Lactose synthesis: the possibilities of regulation. *J. Dairy Sci.* **63**, 328–336 [Review]. doi:10.3168/JDS.S0022-0302(80)82934-1

Levay-Young, B. K., Hamamoto, S., Imagawa, W., and Nandi, S. (1990). Casein accumulation in mouse mammary epithelial cells after growth stimulated by different hormonal and nonhormonal agents. *Endocrinology* **126**, 1173–1182. doi:10.1210/ENDO-126-2-1173

Lkhider, M., Delpal, S., Le Provost, F., and Ollivier-Bousquet, M. (1997). Rat prolactin synthesis by lactating mammary epithelial cells. *FEBS Lett.* **401**, 117–122. doi:10.1016/S0014-5793(96)01450-0

Neville, M. C., McFadden, T. B., and Forsyth, I. (2002). Hormonal regulation of mammary differentiation and milk secretion. *J. Mammary Gland Biol. Neoplasia* **7**, 49–66. doi:10.1023/A:1015770423167

Nishikawa, S., Moore, R. C., Nonomura, N., and Oka, T. (1994). Progesterone and EGF inhibit mouse mammary gland prolactin receptor and beta-casein gene expression. *Am. J. Physiol.* **267**, C1467–C1472.

Oakes, S. R., Rogers, R. L., Naylor, M. J., and Ormandy, C. J. (2008). Prolactin regulation of mammary gland development. *J. Mammary Gland Biol. Neoplasia* **13**, 13–28. doi:10.1007/S10911-008-9069-5

Quirk, S. J., Gannell, J. E., and Funder, J. W. (1982). Progesterone receptors in mammary gland of the pregnant rat. *Endocrinology* **111**, 1883–1890. doi:10.1210/ENDO-111-6-1883

Quirk, S. J., Gannell, J. E., and Funder, J. W. (1986). Adrenocorticoid-dependent alpha-lactalbumin synthesis in rat mammary gland explants: antagonist studies. *Clin. Exp. Pharmacol. Physiol.* **13**, 233–239. doi:10.1111/J.1440-1681.1986.TB00341.X

Rosen, J. M., O'Neal, D. L., McHugh, J. E., and Comstock, J. P. (1978). Progesterone-mediated inhibition of casein mRNA and polysomal casein synthesis in the rat mammary gland during pregnancy. *Biochemistry* **17**, 290–297. doi:10.1021/BI00595A016

Rosenblatt, J. S., Mayer, A. D., and Giordano, A. L. (1988). Hormonal basis during pregnancy for the onset of maternal behaviour in the rat. *Psychoneuroendocrinology* **13**, 29–46 [Review]. doi:10.1016/0306-4530(88)90005-4

Rosenblatt, J. S., Olufowobi, A., and Siegel, H. I. (1998). Effects of pregnancy hormones on maternal responsiveness, responsiveness to oestrogen stimulation of maternal behaviour and the lordosis response to oestrogen stimulation. *Horm. Behav.* **33**, 104–114. doi:10.1006/HBEH.1998.1441

Russo, J., and Russo, I. H. (2008). Breast development, hormones and cancer. *Adv. Exp. Med. Biol.* **630**, 52–56. doi:10.1007/978-0-387-78818-0_4

Salicioni, A. M., Caron, R. W., and Deis, R. P. (1993). Adrenal progesterone facilitates the negative feedback of oestrogen on LH release in ovariectomized rats. *J. Endocrinol.* **139**, 253–258. doi:10.1677/JOE.0.1390253

Shi, W. L., and Zhu, P. D. (1990). Autoradiographic localization of [3H]RU 486 and [3H]progesterone in the uterus, pituitary and hypothalamus of the rat. *Hum. Reprod.* **5**, 505–509.

Siegel, H. I. (1986). Hormonal basis of maternal behaviour in the rat. *Ann. N. Y. Acad. Sci.* **474**, 202–215 [Review]. doi:10.1111/J.1749-6632.1986.TB28012.X

Soaje, M., and Deis, R. P. (1997). Opioidergic regulation of prolactin secretion during pregnancy: role of ovarian hormones. *J. Endocrinol.* **155**, 99–106. doi:10.1677/JOE.0.1550099

Soaje, M., de Di Nasso, E. G., and Deis, R. P. (2002). Regulation by endogenous opioids of suckling-induced prolactin secretion in pregnant and lactating rats: role of ovarian steroids. *J. Endocrinol.* **172**, 255–261. doi:10.1677/JOE.0.1720255

Soaje, M., Valdez, S., Bregonzio, C., Penissi, A., and Deis, R. P. (2006). Dopaminergic mechanisms involved in prolactin release after mifepristone and naloxone treatment during late pregnancy in the rat. *Neuroendocrinology* **84**, 58–67. doi:10.1159/000096825

Stocco, C. O., Chedrese, J., and Deis, R. P. (2001). Luteal expression of cytochrome P450 side-chain cleavage, steroidogenic acute regulatory protein, 3beta-hydroxysteroid dehydrogenase and 20alpha-hydroxysteroid dehydrogenase genes in late pregnant rats: effect of luteinizing hormone and RU486. *Biol. Reprod.* **65**, 1114–1119. doi:10.1095/BIOLREPROD65.4.1114

Telleria, C. M., and Deis, R. P. (1994). Effect of RU486 on ovarian progesterone production at pro- oestrus and during pregnancy: a possible dual regulation of the biosynthesis of progesterone. *J. Reprod. Fertil.* **102**, 379–384. doi:10.1530/JRF.0.1020379

Telleria, C. M., Stocco, C. O., and Deis, R. P. (1995). Luteolytic action of RU486: modulation of luteal 3 beta-hydroxysteroid dehydrogenase and 20 alpha-hydroxysteroid dehydrogenase activities in late pregnant rats. *Journal of Steroid Biochemistry & Mol. Biol.* **52**, 567–573.

Telleria, C. M., Goyeneche, A. A., Cavicchia, J. C., Stati, A. O., and Deis, R. P. (2001). Apoptosis induced by antigestagen RU486 in rat corpus luteum of pregnancy. *Endocrine* **15**, 147–156. doi:10.1385/ENDO:15:2:147

Terkel, J., Witcher, J. A., and Adler, N. T. (1990). Evidence for "memory" of cervical stimulation for the promotion of pregnancy in rats. *Horm. Behav.* **24**, 40–49. doi:10.1016/0018-506X(90)90025-S

Topper, Y. J., and Freeman, C. S. (1980). Multiple hormone interactions in the developmental biology of the mammary gland. *Physiol. Rev.* **60**, 1049–1106.

Vermouth, N. T., and Deis, R. P. (1974). Prolactin release and lactogenesis after ovariectomy in pregnant rats: effect of ovarian hormones. *J. Endocrinol.* **63**, 13–20. doi:10.1677/JOE.0.0630013

Vermouth, N. T., and Deis, R. P. (1975). Inhibitory effect of progesterone on the lactogenic and abortive action of prostaglandin F2alpha. *J. Endocrinol.* **66**, 21–29. doi:10.1677/JOE.0.0660021

Wlodek, M. E., Ceranic, V., O'Dowd, R., Westcott, K. T., and Siebel, A. L. (2009). Maternal progesterone treatment rescues the mammary impairment following uteroplacental insufficiency and improves postnatal pup growth in the rat. *Reprod. Sci.* **16**, 380–390. doi:10.1177/1933719108327592