Pituitary and ovarian response to transient doe–litter separation in nursing rabbits

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The effects of a transient doe–litter separation on plasma prolactin, FSH and oestradiol concentrations, as well as the effect on LH response to exogenous GnRH administered at the time of artificial insemination, were determined in nursing rabbits. The effects on fertility, and litter size after parturition, as well as litter survival after doe–litter separation, were also studied. Control does ($n = 12$) had free access to nursing, whereas biostimulated does ($n = 12$) were separated from their litters for 48 h before artificial insemination. Plasma prolactin concentrations were decreased 24 h after the doe–litter separation ($P < 0.05$). The response of prolactin to suckling reached 10 times the basal values measured on day 10 after parturition ($P < 0.0001$). Increased oestradiol concentrations were found during the 48 h after the doe–litter separation: at 0 h, before artificial insemination ($P < 0.0001$), 1.0–2.0 h after artificial insemination ($P < 0.001$), at 2.5 h ($P < 0.05$), 3.0 h ($P < 0.01$), and at 3.5 h ($P < 0.05$) after artificial insemination. Exogenous GnRH administered at the time of artificial insemination caused a greater LH response in does previously separated from their litters during 48 h ($P < 0.01$). The transient doe–litter separation did not affect plasma FSH concentrations, fertility, litter size or litter survival. These results suggest that a transient separation of nursing does from their litters before artificial insemination results in a decrease in plasma prolactin concentrations that could promote growth of follicular waves, and high steroidogenesis activity, leading to increased oestradiol concentrations and inducing higher sensitivity of the pituitary gland to exogenous GnRH. These findings associated to the absence of suckling episodes would lead to higher LH response and, therefore, exert a major effect on fertility.

Introduction

In nursing rabbits, the sexual receptivity and fertility obtained after artificial insemination appear to be depressed during the period of lactation, through a hormonal antagonism between prolactin and release of gonadotrophins (Theau-Clément and Roustau, 1992; Fortun and Bolet, 1995; Ubilla and Rebollar, 1995). In addition, high prolactin concentrations could inhibit the ovulatory process (Lin *et al.*, 1987) and thus affect the rupture of mature Graafian follicles (Yoshimura *et al.*, 1990). Nursing rabbits that accept mating showed higher fertility and litter size compared with non-receptive rabbits (Rebollar *et al.*, 1992). In rabbits, it has been established that mating is directly related to the turgidity and colour of the vulva (Rodríguez *et al.*, 1989). Three sexual receptivity levels have been described: does with red or pink turgid vulvas have 92% mating acceptance (high sexual receptivity), does with pink non-turgid or purple turgid vulvas have 55% mating acceptance (medium sexual receptivity), and does with purple, non-turgid, or pale vulvas have 24% mating acceptance (low sexual receptivity)

(Rodríguez *et al.*, 1989). Higher ovulation responses are obtained in rabbits showing high or medium sexual receptivity after i.m. injection of 20 µg or 40 µg GnRH (Rodríguez and Ubilla, 1988), with a clear LH dose–response to GnRH in medium and high sexual receptivity lactating rabbits (Rodríguez *et al.*, 1989). Highest LH and FSH concentrations are reached 60–90 min after exogenous administration of GnRH, decreasing to basal concentrations 4–5 h later (Mills and Gerardot, 1984; Rodríguez *et al.*, 1989; Rebollar *et al.*, 1997).

Mean plasma concentrations of oestradiol showed two increments, one during the first days and a second increment during the last week (day 24–30), in high sexual receptivity does; in contrast medium or low sexual receptivity lactating rabbits did not follow this pattern during lactation (Ubilla and Rebollar, 1995). High plasma concentrations of prolactin associated with low plasma oestradiol and sexual receptivity are observed from days 7 to day 24 of lactation (Ubilla *et al.*, 1992; Ubilla and Rebollar, 1995). All of these changes induce an irregular alternation of oestrus and anoestrus in rabbits which make it difficult to establish a synchronized reproduction rhythm using artificial insemination (Maertens, 1998).

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Several methods for the synchronization of oestrus have been proposed to improve fertility in nursing rabbits: these include systematic stimulation of ovarian activity with gonadotrophins (Colin, 1992), or administration of PGF_{2α} (Dragan *et al.*, 1996; Pimienta *et al.*, 1996). More recently, several alternative methods to hormonal treatments based on a controlled lactation (the so-called biostimulation methods) have been developed to stimulate the sexual receptivity and fertility in rabbits. Separation of does from litters for short periods before artificial insemination resulted in higher sexual receptivity and fertility (Pavois *et al.*, 1994; Alvaríño *et al.*, 1998; Castellini *et al.*, 1998; Maertens, 1998). In addition, changes in the environment of the rabbit account for higher sexual receptivity and fertility (Luzi and Crimella, 1998). After biostimulation, fertility reached similar values to those obtained after administering PMSG before artificial insemination (Facchin *et al.*, 1992; Pavois *et al.*, 1994). These stimuli probably promote sexual receptivity, and increase fertility through changes in the activities of the hypothalamus–pituitary–ovarian axis (Castellini *et al.*, 1998). Nevertheless, the endocrine mechanisms involved in these events are not fully understood.

Thus, the aim of this study was to investigate the effects of a transient doe–litter separation on plasma prolactin, FSH and oestradiol concentrations, as well as the effect on LH response to exogenous GnRH administered at the time of artificial insemination in nursing rabbits. The effects on fertility and litter size after parturition, as well as litter survival after doe–litter separation, were also examined.

Materials and Methods

Animals

Adult, multiparous, lactating, crossbreed rabbits (New Zealand White × Californian; aged 12–18 months; 3 870 ± 85 g body weight) ($n = 24$) raised in the Animal Production Department, maintained under controlled light–dark cycles (16 h light: 8 h dark), and housed in individual metal cages (32 cm × 52 cm) were used in this study. The rabbits had free access to a commercial diet (Lab Rabbit Chow, Purina Mills Inc. (Torrejón de Ardoz-Madrid): 16.2% crude protein, 2.5% fat and 13.5% crude fibre), and tap water. On day 1 after parturition, the size of the litters was standardized to 8–9 by adding or removing pups.

Experimental design

This study was performed according to the CEE Council Directive (86/609, 1986) for the care of experimental animals.

Lactating does were randomly allocated to two groups: control ($n = 12$) or biostimulated ($n = 12$). Control does had free access to nursing, whereas biostimulated does were separated from their litters by a metal screen for 48 h before artificial insemination (Alvaríño *et al.*, 1998; Luzi and Crimella, 1998) from day 9 (at 09:00 h) until day 11 (at 09:00 h) of the lactation period. The nest boxes of biostimulated does were opened at 09:00 h on day 11 of lactation to enable nursing of the litters.

Artificial insemination

Biostimulated does were artificially inseminated on day 11 of lactation immediately after collection of blood samples and nursing. Control does were inseminated on day 11 of lactation after collection of blood samples. Artificial inseminations were carried out using 1 ml of fresh semen diluted with a commercial extender (MA 24, Ovejero Lab., León). Each dose contained at least 25×10^6 spermatozoa ml⁻¹. Ovulation was induced by Gonadoreline, a synthetic analogue of GnRH (20 µg per rabbit i.m.; Ovejero Lab., León), (Alvaríño *et al.*, 1998).

Collection of blood samples

Separation of does from litters was performed at 09:00 h on day 9 of the lactation period. This was considered as the start point for the collection of blood samples in both groups of does. Blood samples (3 ml) were collected to determine the profile of plasma prolactin, oestradiol and FSH concentrations, as well as plasma LH pattern release in response to exogenous GnRH administered at the time of artificial insemination. Samples were collected from both groups from the margin ear vein into heparinized tubes, and immediately centrifuged at 1000 g for 10 min, at 8°C. Plasma was stored at –20°C until analysed.

Blood samples were collected at intervals of 24 h, from day 9 to day 11 of lactation, to analyse plasma prolactin, oestradiol, FSH and LH concentrations, before artificial insemination and GnRH administration. The first sample on day 11 of lactation was collected before artificial insemination and GnRH administration (at 0 h) in the control group, and just after nursing and before artificial insemination and GnRH administration (at 0 h) in the biostimulated group.

For determination of plasma oestradiol, FSH, and LH concentrations after artificial insemination and GnRH administration, collection of blood samples was performed in both groups 1.0 h after artificial insemination, and thereafter, at intervals of 30 min for 4 h, and finally at 7 h after artificial insemination.

Both groups of does nursed their litters at least 3 h before collection of blood samples on day 9 for determination of basal plasma concentrations of prolactin. Control rabbits nursed their litters at least 3 h before collection of blood samples on day 10 and 11 of the lactation. No does of either group nursed their litters after artificial insemination until the last blood sample was collected 7 h later.

Pregnancies were diagnosed by abdominal palpation on day 12 after insemination. Fertility and litter size was determined after parturition. The effect on litter survival was periodically determined after the doe–litter separation.

Hormone analysis

Plasma LH, FSH and prolactin concentrations were measured in duplicate samples by specific radioimmunoassay methods, using AFP-3120489, AFP-472176 and AFP-991086 antibodies for LH, FSH and prolactin, respectively, supplied by

the National Institutes of Health (NIH, Bethesda, MD), and A. F. Parlow (Harbour-UCLA Medical Centre, CA). Hormones were labelled with ^{125}I by the Chloramine-T-method (Greenwood *et al.*, 1963). The antibody titres used were 1:250 000 for LH, 1:45 000 for FSH and 1:62 500 for prolactin assays, respectively. The volume of plasma used was 100 μl for LH, 75 μl for FSH, and 10 μl for prolactin determinations. *Staphylococcus aureus*, prepared by the Universidad Autónoma, Departamento de Fisiología Vegetal (UAM, Madrid), was used to precipitate the bound fraction. The assays were previously validated in our laboratory (Ubilla *et al.*, 1992).

All samples were measured in the same radioimmunoassay to avoid inter-assay variations. The limits of detection for LH, FSH and prolactin were 0.05, 0.48 and 0.125 ng ml^{-1} , respectively. The intra-assay coefficients of variation were <5% for LH, FSH and prolactin. Plasma concentrations of oestradiol were determined using a commercial ^{125}I RIA kit (ICN Pharmaceuticals, Inc. Diagnostics Division Costa Mesa, CA, Lot No. E2K9917). Oestradiol concentrations were expressed as pg ml^{-1} . All samples were analysed in the same assay. The limit of detection was 0.74 pg ml^{-1} , and the intra-assay coefficient of variation was <6%.

Statistical analysis

All analyses were conducted using the Statistical Analysis System Software for Windows, version 6.12 (SAS Institute Inc., NC). A non-parametric procedure (PROC CATMOD; SAS, 1990) was used to identify significant effects of treatment on the variables that did not present a normal distribution (LH, FSH, prolactin, oestradiol concentrations and fertility). The means were compared using the contrast statement. Catmod is a procedure for categorical data modelling. Catmod analyses data that can be represented by a contingency table by fitting linear models to functions of response frequencies and can be used for linear modelling. The contrast statement constructs and tests linear functions of the parameters. One-way analysis of variance (ANOVA) using the general linear models (GLM) procedure of SAS (1990) was used to identify significant effects of treatment on the litter size. This procedure performs analysis of variance for balanced data. The means were compared using Duncan's multiple range test (SAS, 1990). Values were considered significant if $P < 0.05$. Data are presented as mean $\pm$ SEM.

Results

Prolactin

There were no differences in plasma concentrations of prolactin 48 h before artificial insemination between the control and biostimulated group. Plasma concentrations of prolactin of biostimulated does were lower 24 h after doe–litter separation, compared with previous values of the same group, and with control animals ($P < 0.05$). Plasma prolactin concentrations were higher in biostimulated

rabbits after nursing compared with those of the control group ($P < 0.0001$; Fig. 1).

Oestradiol

Mean plasma concentrations of oestradiol were similar among control does. There were no differences between the two groups at 48 h and 24 h before artificial insemination. The oestradiol concentrations of the biostimulated does were higher than the previous values of the same group from 0 h to 2.0 h after artificial insemination ($P < 0.001$). The oestradiol concentrations of the biostimulated group were higher than those of the control group during the following periods: at 0 h ($P < 0.0001$), 1.0–2.0 h after artificial insemination ($P < 0.001$), at 2.5 h ($P < 0.05$), 3.0 h ($P < 0.01$), and 3.5 h ($P < 0.05$) after artificial insemination. Similar plasma concentrations of oestradiol were observed in both groups from 4 h to 7 h after artificial insemination (Fig. 2).

LH

Plasma LH concentrations of both groups of rabbits were similar in samples collected at 48 h, 24 h and 0 h before artificial insemination, and at 3–7 h after artificial insemination. Plasma LH concentrations from 1.0 h to 2.5 h after GnRH administered at the time of artificial insemination were higher in both groups compared with the basal values of each group (control: $P < 0.0001$; biostimulated $P < 0.0005$). Plasma concentrations of LH were higher in biostimulated does at 1 h after exogenous GnRH and artificial insemination than in control does ($P < 0.01$), and at 1.5–2.5 h after artificial insemination ($P < 0.005$) compared with the control group (Fig. 3).

Fig. 1. Plasma prolactin concentrations in control doe rabbits (□) ($n = 12$), and in biostimulated doe rabbits (■) ($n = 12$) before artificial insemination. Biostimulated does nursed their litters before artificial insemination and sampling. The arrow indicates the time of artificial insemination and GnRH administration. The values are means $\pm$ SEM. Significant differences between groups are indicated by different letters: small letters = ($P < 0.05$); capital letters = ($P < 0.0001$).

Fig. 2. Plasma oestradiol concentrations in control doe rabbits (○) ($n = 12$), and in biostimulated doe rabbits (□) ($n = 12$). Biostimulated does nursed their litters before artificial insemination and sampling. The arrow indicates the time of artificial insemination and GnRH administration. Values are means $\pm$ SEM. Asterisks indicate differences between groups: **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. Significant differences compared with basal concentrations are indicated by different letters ($P < 0.001$). Mean plasma oestradiol concentrations were similar among control does.

Fig. 3. Plasma LH concentrations in control doe rabbits (○) ($n = 12$) and in biostimulated doe rabbits (□) ($n = 12$). Biostimulated does nursed their litters before artificial insemination and sampling. The arrow indicates the time of artificial insemination and GnRH administration. Values are means $\pm$ SEM. Asterisks indicate differences between groups: *** $P < 0.005$; ** $P < 0.01$. Control and biostimulated does showed higher concentrations 1 h to 2.5 h after artificial insemination compared with the concentrations before artificial insemination for each group ($P < 0.0001$).

FSH

There were no significant differences in the mean plasma FSH concentrations between the two groups of animals, or among control or biostimulated does ($P < 0.1$; Fig. 4).

Fig. 4. Plasma FSH concentrations in control doe rabbits (○) ($n = 12$) and in biostimulated doe rabbits (□) ($n = 12$). Biostimulated does nursed their litters before artificial insemination and sampling. The arrow indicates the time of artificial insemination and GnRH administration. Values are means $\pm$ SEM. Biostimulation did not alter plasma FSH concentrations.

Fertility, litter size and litter survival

No differences were observed in the fertility and litter size between biostimulated and control rabbits (100% versus 83.3%, and 9.08 ± 0.41 versus 9.2 ± 0.39 , respectively; $P < 0.1$). All of the litters survived after the transient doe–litter separation.

Discussion

The results of the present study indicate that changes in plasma concentrations of oestradiol and prolactin after doe–litter separation and in the pituitary LH response to exogenous GnRH administered at the time of artificial insemination may explain the higher ovarian stimulation and pituitary responsiveness after biostimulation. Plasma concentrations of prolactin measured in control does on days 9, 10 and 11 of lactation, and on day 9 in biostimulated rabbits are in agreement with those observed 2–3 h after one suckling episode a day during mid-lactation in rabbits (McNeilly and Friesen, 1978; Fuchs *et al.*, 1984), and is characterized by sustained high plasma concentrations of prolactin after suckling, which decline to basal concentrations (Fuchs *et al.*, 1984; Ubilla *et al.*, 1992). Plasma concentrations of prolactin decreased 24 h after the doe–litter separation, probably as a result of the absence of suckling episodes which stimulate prolactin release (McNeilly and Friesen, 1978). The transitory hypoprolactinaemia found in these does may be implicated in the stimulatory effects on ovarian activity, and sexual receptivity, before artificial insemination. Ubilla *et al.* (1992) demonstrated that high plasma concentrations of prolactin can inhibit the oestrogenic effect on sexual receptivity during the first week of lactation. Similarly, Theau-Clement *et al.* (1990) observed that lactation and nursing have a depressive

effect on sexual behaviour. Hyperprolactinaemia can alter follicular maturation and steroidogenesis in humans (Jacobs *et al.*, 1976; Kauppila *et al.*, 1982), and can inhibit the hypothalamic–pituitary axis, suppressing the gonadotrophin secretion, and follicular development (McNeilly *et al.*, 1982; McNeilly, 1987), not only in rabbits but also in rats (Esquifino *et al.*, 1984). The response to suckling was an increase in prolactin to ten times the basal values measured on day 10 after parturition, and is of similar magnitude to that reported by McNeilly and Friesen (1978) after mechanical stimulation of the nipples. The hypoprolactinaemia together with the increased plasma concentrations of oestradiol found in the present study explain the greater maturation of follicles growing in waves after parturition (Diaz *et al.*, 1987). Indeed, increased follicular steroidogenic activity was observed during the first postpartum day (Osteen and Mills, 1980) and plasma concentrations of oestradiol were high on day 1 and 8 postpartum (Rebollar *et al.*, 1992). Increased oestradiol concentrations were evident 48 h after separation of the does from their litters, before artificial insemination and GnRH administration. The concentrations of oestradiol found during this period were comparable with those previously described, and could be related to high follicular activity (Rebollar *et al.*, 1992; Ubilla and Rebollar, 1995).

Administration of GnRH resulted in an LH response in both groups of does. The plasma concentrations of LH obtained were comparable with those observed by Carlson *et al.* (1977) and Rebollar *et al.* (1997). Exogenous GnRH administered at the time of artificial insemination resulted in a greater LH response in does previously separated from their litters for 48 h. This effect could be related to the higher concentrations of oestradiol found in biostimulated does before artificial insemination, enhancing GnRH-stimulated release of LH, as was reported by Huang and Miller (1980). In sheep, oestradiol increases the sensitivity of the pituitary gland to GnRH by stimulating the expression of the GnRH receptor gene that leads to an increased number of GnRH receptors (Laws *et al.*, 1990; Wu *et al.*, 1994; Turzillo *et al.*, 1994). These effects are probably important when oestradiol concentrations are increased in developing ovarian follicles in the absence of the influence of luteal progesterone (Turzillo *et al.*, 1998). In rabbits, as in other species, the secretion of GnRH by the hypothalamus, and thus LH, appeared to be controlled by ovarian steroids, and also probably by endogenous opioids (Orstead and Spies, 1987). McNeilly (1988) suggested that the inhibition of LH during lactation might be a result of an increased secretion of β -endorphin produced by suckling in ewes. However, in the present study β -endorphin should have been decreased, as suckling stimuli were absent in biostimulated animals. There was no luteal influence, as all does kindled 9 days before the beginning of the experiment.

The high concentrations of oestradiol together with the absence of suckling episodes and a transitory hypoprolactinaemia before exogenous GnRH and artificial insemination could have a relevant influence on the LH response of the biostimulated does. Although administration of GnRH and artificial insemination were performed after suckling, the increased LH response observed indicates that the stimulatory effects of doe–litter separation on the ovaries

and pituitary gland could have been produced before the GnRH administration and artificial insemination.

This study failed to show a significant effect of biostimulation on plasma FSH concentrations, probably due to the high variation of the values obtained. Litter size and litter survival were not affected by the doe–litter separation, and were consistent with previous results (Alvariño *et al.*, 1998).

Although no significant differences in fertility were obtained, possibly due to the small number of animals inseminated, the results of this study indicate that the increased breeding efficiency obtained in farm conditions using a large number of lactating does separated from their litters for short periods before artificial insemination (Alvariño *et al.*, 1998; Castellini *et al.*, 1998; Maertens 1998) could result from several stimulatory actions and endocrine changes, probably triggered by transitory hypoprolactinaemia, and the absence of suckling episodes.

In summary, the results of the study reported here indicate that a transient separation of nursing does from their litters before artificial insemination leads to a decrease in plasma concentrations of prolactin that could promote growth of follicular waves, and high steroidogenesis leading to increased plasma concentrations of oestradiol that induce an increased sensitivity of the pituitary gland to exogenous GnRH. These findings, associated with the absence of suckling episodes, and thus, probably low endogenous opioid secretion would lead to a higher LH response and, therefore, exert a major effect on fertility. Additional studies on experimental protocols involving changes in specific hormone concentrations during and after biostimulation would help determine the role of the different hormones on the reproductive performance of doe rabbits.

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