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Original article

Effect of the increase of steroid binding plasma levels after passive immunization against testosterone on the control of luteinizing hormone (LH) secretion in ovariectomized underfed dairy heifers

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Summary — The ability of passive immunization against testosterone to increase sex steroid binding levels in plasma and thus to overcome the negative feedback of oestradiol-17β (E_2) on LH secretion in underfed heifers was investigated. Dairy heifers were ovariectomized and divided in 3 groups: high energy diet (H group, $n = 4$), low energy diet (L group, $n = 3$) and low energy diet + E_2 implants (LE_2 group, $n = 4$). Twenty-four h before injection of bovine immunoglobulins, the mean concentrations of LH were not different between H and L groups. LH baseline was lower (0.8 *vs* 1.1 ng/ml, $P < 0.03$) and the median number of LH pulses was higher (10 *vs* 5, $P < 0.03$) in H than in L group. E_2 markedly decreased ($P < 0.01$) the mean and basal concentrations of LH (0.27 ng/ml), and number of LH pulses (0) in the LE_2 group ($P < 0.05$). After injection of anti-testosterone immunoglobulins in the L group, mean and basal LH concentrations tended to decrease. The median number of LH pulses in the L group rose 8 days after immunization (5 *vs* 7 on day −1 and day +8, $P \leq 0.05$). Amplitude of pulses tended to decrease after injection ($P = 0.08$). In the LE_2 group, the mean concentration and baseline of LH were not affected by passive immunization against testosterone, while pulses of LH appeared at day +1 and rose ($P = 0.07$) at day +8 after immunization with 3.5 pulses. Thus passive immunization against testosterone increased sex steroid binding levels in plasma of underfed heifers and reduced the amount of E_2 and/or non-ovarian steroids available for negative feedback effects on LH secretion.

passive immunization / steroid / LH / underfeeding / heifers

Résumé — Effet de l'augmentation du niveau de liaison plasmatique des stéroïdes après immunisation passive contre la testostérone sur le contrôle de la sécrétion de l'hormone lutéinisante (LH) chez les génisses laitières ovariectomisées et sous-alimentées. *La possibilité d'augmenter les niveaux de liaison plasmatiques des stéroïdes par l'immunisation passive contre la testostérone (IP), et donc de bloquer le rétro-contrôle négatif de l'œstradiol-17β (E_2) sur la sécrétion de la LH chez des génisses sous-alimentées, a été étudiée. Des génisses laitières pubères ont été ovariectomisées et réparties en 3 groupes : régime alimentaire à haut niveau énergétique (groupe H,* n = *4), à faible niveau énergétique (groupe L,* n = *3) et à faible niveau énergétique associé à des implants d'E_2 (groupe LE_2,* n = *4). Vingt-quatre heures avant l'injection des immunoglobulines bovines, le niveau de base de LH était plus faible (0,8* vs *1,1 ng/ml,* P < *0,03) et le nombre médian de*

pulses de LH plus élevé (10 vs 5, P < 0,03) pour le groupe H que pour le groupe L. E_2 a diminué fortement (P < 0,01) les concentrations moyennes et basales de LH dans le groupe LE_2 et supprimé la pulsatilité de LH (P < 0,05). L'injection d'immunoglobulines anti-testostérone au groupe L a eu tendance à diminuer les concentrations moyennes et basales de LH. Le nombre médian de pulses de LH a augmenté dans le groupe L 8 jours après IP (5 vs 7 aux jours −1 et +8, P ≤ 0,05), cette augmentation s'est ensuite maintenue; l'amplitude des pulses a, semble-t-il, diminué après IP (P = 0,08). Dans le groupe LE_2 il n'y a pas eu, après IP, de modification des concentrations moyennes et du niveau de base de LH mais des pulses de LH sont apparus à j + 1 et ont augmenté (P = 0,07) à j + 8 avec 3,5 pulses. En conclusion, IP a augmenté les niveaux de liaison plasmatique des stéroïdes chez les génisses sous alimentées et a réduit la quantité de E_2 et/ou de stéroïdes non-ovariens disponibles pour exercer un rétro-contrôle négatif sur la sécrétion de LH.

immunisation passive / stéroïde / LH / sous-alimentation / génisse

INTRODUCTION

Failure of the bovine female to show ovarian activity during the breeding period is a primary cause of decreased reproductive performance in beef cattle (Wiltbank, 1970). Restricting dietary intake during late pregnancy and the beginning of lactation in cow, delays the first *post-partum* ovulation by reducing LH and FSH levels (Echternkamp *et al*, 1982; Terqui *et al*, 1982; Gauthier *et al*, 1983). Oestradiol-17β (E_2) is known to suppress LH secretion in prepubertal (Day *et al*, 1984) and *post-partum* anoestrus bovine females (Acosta *et al*, 1983). Heifers that were anoestrus due to restriction of intake of dietary energy had an increased sensitivity to negative feedback of E_2, which resulted in a decreased secretion of LH (Imakawa *et al*, 1986). The secretion of LH in ovariectomized heifers maintained on nutritionally inadequate diets is more sensitive to inhibitory effects of E_2 than in the same animals fed nutritionally adequate diets (Imakawa *et al*, 1987). There is a sex steroid-binding-protein (SBP) in the plasma of cattle, which binds mainly 5α-dihydrotestosterone and testosterone, but also shows some cross-reactivity with E_2 (Martin *et al*, 1976; Suzuki *et al*, 1977; Lermite and Terqui, 1991). It was recently observed that plasma SBP levels were decreased by underfeeding in ovariectomized heifers (Lermite and Terqui, 1991). This suggested that a decrease in sex steroid binding levels in plasma of underfed heifers may contribute to an apparent increase in sensitivity to E_2 feedback. To test this hypothesis, underfed ovariectomized heifers, with and without E_2 replacement, were passively immunized with antisera that showed cross-reactivity with E_2 similar to that of SBP. An increase in LH secretion after immunization was anticipated if steroid binding levels in plasma is a contributing factor to apparent changes in feedback sensitivity.

MATERIALS AND METHODS

Preparation of anti-testosterone bovine immunoglobulins

The antiserum was raised in castrated dairy bulls (Friesian x Holstein) and in intact dairy and beef cows (Friesian x Holstein and Charolais), using a multi-intradermal injection of testosterone-3-human serum albumin conjugate and Freund's complete adjuvant. After several booster immunizations, blood was collected and serum was treated (Institut Mérieux, France): sera from the different animals were pooled and a

gamma globulin-enriched fraction was obtained by $(NH_4)_2SO_4$ precipitation (50% saturation). The precipitate was dissolved in a minimum of water with NaCl (5 g/l) and glycocoll (10 g/l) at pH 7. The final solution, which was concentrated by about 6-fold, was then sterilized. Normal bovine immunoglobulins (without anti-testosterone activity) were prepared using the same procedure.

Animals

Post-pubertal dairy heifers (Friesian x Holstein), 18-months old were used in this study. They averaged 415 ± 34 kg body weight (mean ± SD) at the beginning of the experiment. All heifers were weighed before the time of feeding on 2 consecutive days and at 2-week intervals throughout the experiment; the average weight of the 2 consecutive days was used to determine weight changes.

Experimental design

Heifers were assigned to either a high energy diet (10.1 Mcal net energy/animal/day, n = 4 heifers, H group) or a low energy diet (3.7 Mcal net energy/animal/day, n = 7 heifers, L group). All heifers were ovariectomized 87 days after the beginning of the experimental diets. Four of the L heifers received 3 silastic implants each (id = 0.335 cm; od = 0.465 cm; length = 7 cm; Dow Corning, France) filled with oestradiol-17β (Roussel-Uclaf, France). They were inserted subcutaneously in the shoulder blade at the time of ovariectomy (n = 4, LE_2 group). The remaining heifers (L group, n = 3 and H group, n = 4) were not implanted. By day 185 after ovariectomy L and LE_2 animals received a single subcutaneous injection of anti-testosterone bovine immunoglobulins, while H group heifers were injected with normal bovine immunoglobulins.

Daily blood samples were collected throughout the experimental period to follow anti-testerone immunoglobulin titres after injection. To determine secretory patterns of LH, serial blood samples were collected by jugular venipuncture at 10-min intervals for a 6-h period on days (D) −1, +1, +3, +8, +20, +30 after injection of immunoglobulins (D0); plasma was harvested and stored at −20 °C until assayed. E_2 concentration was determined for all the heifers in one sample of each serial blood collection.

Anti-testosterone bovine immunoglobulins characterization

Titre, specificity and affinity constant of bovine anti-testosterone immunoglobulins were measured. The titre was estimated by the addition of 0.1 ml of diluted antiserum to 0.1 ml of 0.1 M phosphate gelatin assay buffer (0.1% gelatin in phosphate buffer solution, pH = 7) with 0.1 ml of a fixed mass of [^{3}H]-labeled testosterone (180 pg, [1, 2, 6, 7-^{3}H] testosterone, specific activity 3.25 TBq/mmol; Amersham, UK). The reaction volume was then incubated overnight at 4 °C. The free fraction was separated by addition of 1 ml of a suspension in assay buffer of dextran T-70 (250 mg/l) and charcoal (1 g/l) and incubated for 12 min at 4 °C before centrifugation (1 500 *g*, 15 min). The supernatant containing bound steroid was transferred into vials and radioactivity was determined using a liquid scintillation spectrometer. The titre of antiserum was defined as the amount of tritiated testosterone bound by litre of antiserum or by litre of plasma of passively immunized animals (nmol testosterone bound/l). The titre of the bovine anti-testosterone immunoglobulins used in this study was 14 300 nmol of testosterone bound by litre of antiserum.

The specificity of anti-testosterone bovine immunoglobulins was tested by performing cross-reactivity studies with various radioinert steroids (Steraloid, USA). The percentage of cross-reaction was defined by the ratio of the amount of unlabeled testosterone which gave 50% of displacement of the [^{3}H]-testosterone to the amount of the competitor steroid which gave 50% of displacement of the [^{3}H]-testosterone. Cross-reactivity was as follows: 5α-dihydrotestosterone, 44%; 5α-androstan-3α, 17β-diol, 22%; androstenedione, 6%; oestradiol-17β, 0.5%; progesterone, < 0.1%; cortisol < 0.1%. The affinity constant (K_a) analyzed using a Scatchard plot (Scatchard, 1949) was 4.0 ± 1.2 x 10^9 M^{-1}.

In order to assess the ability of the anti-testosterone serum to modify steroid feedback in cyclic cows, 40 ml of a first batch of anti-testosterone bovine serum per cow (1004 nmol

of bound testosterone) was injected during the luteal phase, 3 days before prostaglandin analogue injection. LH surge was suppressed in 2 out 4 treated cows, which indicated that anti-testosterone serum inhibited ovulation by neutralizing the positive feedback of endogenous E_2. This was also used to define a "standard" dose for passive immunization, *ie* 2.6 nmol of testosterone bound per kg of average body weight, which represents 69 ± 5.8 ml of the second batch of anti-testosterone bovine immunoglobulins injected in heifers in this study.

Hormone assays

LH concentrations were measured by a double antibody-radioimmunoassay developed by Pelletier *et al* (1982) in sheep and modified by Montgomery *et al* (1985). All samples were included in the same assay to avoid inter-assay variation. The limit of detection was 0.1 ng/ml and the intra-assay coefficient of variation was 9.4% and 11.5% for 0.4 and 4 ng/ml, respectively. Serial blood samples were analyzed to determine mean and basal concentrations (ng/ml) of LH, pulsatility (pulses/6 h) and amplitude (ng/ml) of pulses of LH, through the use of Pulsar algorithms developed by Merriam and Watchter (1982). Plasma E_2 was measured using a double antibody radioimmunoassay (Terqui, 1978) after extraction from plasma by methylenedichloride. The limit of detection was 1.25 pg/ml and the intra-assay coefficient of variation was 9% for 10 pg/ml.

Statistical analysis

Data for body weights, LH and E_2 concentrations and antiserum titres were subjected to a analysis of variance (Kobilinsky and Decoux, 1986). LH pulsatility was assessed by non-parametric test (Siegal, 1956): intra-group variation of LH pulsatility was analyzed using a Wilcoxon test; inter-group variation of LH pulsatility was analyzed using a Mann–Whitney test.

RESULTS

Body weight variation

Following the initiation of feeding the 2 experimental diets, evolution of the mean body weights was different between the 2 nutritional groups of heifers ($P < 0.01$). A weight loss was observed in heifers fed the low energy diet (fig 1). After the initial body weight loss, heifers stabilized in a body weight until the beginning of the blood sampling period. At D -1, H group weighed 561 ± 84 kg and had gained 148 ± 36 kg (36% of initial weight, $P < 0.05$), while L and LE_2 groups weighed 379 ± 32 kg and had lost 38 ± 29 kg (9% of initial weight, $P < 0.05$).

Immunoglobulins titre after passive immunization

Injection of anti-testosterone bovine immunoglobulins into L and LE_2 groups resulted

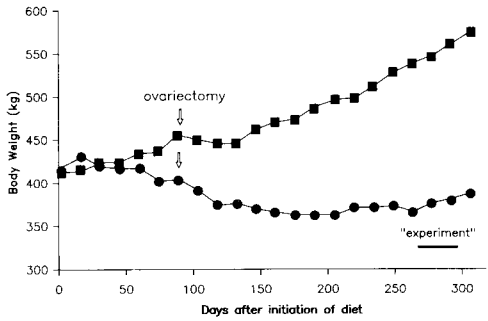

Fig 1. Mean body weights in heifers fed diets that varied in energy (H heifers: high energy —■—; L + LE_2 heifers: low energy —●—). "experiment" : passive immunization + serial blood samples.

in an immediate increase ($P < 0.01$) in tritiated testosterone binding in plasma, relative to H group (fig 2). The binding level reached a maximum 8 days after immunization (44 ± 6 *vs* 15 ± 2 nM at D +8 and D0, respectively) and was still elevated up to day 12. Thereafter it was followed by a low and regular decrease of the binding. Tritiated testosterone binding in plasma of the H group was low and constant throughout this period (13 ± 1.5 nM).

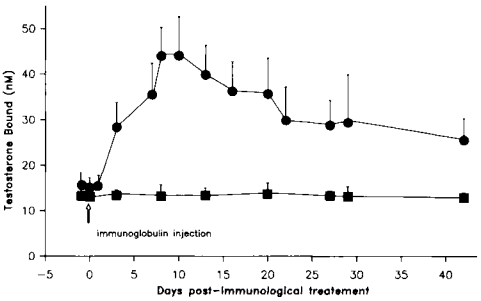

Fig 2. Tritiated testosterone bound *in vitro* (nM, mean and sd) by plasma from non-immunized (H heifers —■—) and testosterone immunized heifers (L + LE_2 heifers —●—) for a 42-d period after treatment.

E_2 plasma levels

Exogenous E_2 administered *via* the silastic implants increased ($P < 0.05$) the concentration of E_2 in jugular blood plasma of LE_2 compared to L and H groups (4 ± 1.7, 1 ± 0.1 and 0.3 ± 0.1 ng/ml in LE_2, L and H groups, respectively). However, concentrations of E_2 were higher in L than in H group ($P < 0.05$). Injection of anti-testosterone immunoglobulins did not modify these concentrations.

Table I. Influence of passive immunization against testosterone on the mean concentrations of LH (ng/ml)[a] in plasma of high fed heifers (H heifers) and underfed heifers with E_2 (LE_2) or without E_2 (L).

Group of heifers	*Days from immunological treatment* −1	+3	+30
H ($n = 4$)	$1.0^b \pm 0.2$	$1.1^b \pm 0.2$	$0.8^b \pm 0.2$
L ($n = 3$)	$1.3^b \pm 0.3$	$1.2^b \pm 0.2$	$0.9^b \pm 0.2$
LE_2 ($n = 4$)	$0.3^c \pm 0.1$	$0.3^c \pm 0.1$	$0.3^c \pm 0.2$

[a] mean ± SD. ($^{b, c}$) Values with different letters are significantly different ($P = 0.05$).

LH plasma levels

At D -1 the mean concentrations of LH in plasma (table I) were not different between H and L groups, whereas LH baseline (table II) was lower ($P < 0.03$) by 27% in H than in L group. The median number of LH pulses (table III) was twice as high ($P < 0.03$) in H than in L group. Amplitude of LH pulses (table IV) tended to be higher in L than in H group ($P = 0.07$). E_2 decreased mean concentration and baseline of LH in the E_2 group ($P < 0.01$), and LH pulses were absent ($P < 0.05$).

Effect of passive immunization on LH plasma levels

After injection of non-specific immunoglobulins in H group, mean concentration, baseline and pulsatility of LH remained constant throughout the 30 days of the experiment. Injection of anti-testosterone immunoglobulins in the L group tended to decrease mean concentration and baseline of LH on D +30 ($P = 0.01$ and $P = 0.06$ for

mean concentration and baseline, respectively). LH pulsatility rose on D + 8 with the median number of pulses being higher ($P \leq 0.05$) than on D -1. This increase was sustained up to D +30. Amplitude of LH pulses tended to decreased after passive immunization on D +20 ($P = 0.08$). In the LE_2 group the low baseline and mean concentrations of plasma LH were not affected by passive immunization against testosterone, but LH pulsatility appeared on D +1 as pulses of low amplitude and this pulsatility tended to rise on D +8 ($P = 0.07$). The median number of LH pulses decreased thereafter related to decrease in antibody titres.

Table II. Influence of passive immunization against testosterone on the basal concentration of LH (ng/ml)[a] in plasma of high fed heifers (H) and underfed heifers with E_2 (LE_2) or without E_2 (L).

Group of heifers	*Days from immunological treatment*		
	−3	*+3*	*+30*
H ($n = 4$)	$0.8^{b} \pm 0.1$	$0.8^{b} \pm 0.1$	$0.6^{bc} \pm 0.1$
L ($n = 3$)	$1.1^{c} \pm 0.2$	$1.0^{c} \pm 0.1$	$0.7^{c} \pm 0.2$
LE_2 ($n = 4$)	$0.3^{d} \pm 0.1$	$0.3^{d} \pm 0.1$	$0.3^{d} \pm 0.2$

[a] mean ± SD. ([b, c, d]) Values with no common letters are significantly different ($P < 0.05$).

DISCUSSION AND CONCLUSION

The injection of anti-testosterone immunoglobulins increased testosterone binding levels in plasma of underfed ovariectomized heifers. It was recently observed that plasma SBP binding capacity was decreased by underfeeding in ovariectomized heifers (Lermite and Terqui, 1991). The levels of binding sites in immunized underfed heifers was 29 nM due to anti-testosterone immunoglobulins 8 days after injection, plus 27 nM of binding sites due to SBP (Lermite and Terqui, 1991). The total was close to 40 nM of SBP binding sites found in high fed heifers (Lermite and Terqui, 1991). Passive immunization would compensate the lack of SBP binding capacity.

Table III. Influence of passive immunization against testosterone on the number of LH pulses[a] in plasma of ovariectomized high fed heifers (H) and underfed heifers with E_2 (LE_2 heifers) or without E_2 (L).

Group of heifers	*Days form immunological treatment*					
	−1	*+1*	*+3*	*+8*	*+20*	*+30*
H ($n = 4$)	10^{b}	10^{b}	10.5^{b}	10^{b}	10.5^{b}	10.5^{b}
L ($n = 3$)	5^{c}	5^{c}	5^{c}	7^{e}	7^{c}	8^{e}
LE_2 ($n = 4$)	0^{d}	1^{d}	2.5^{d}	3.5^{d}	1^{d}	0^{d}

[a] Median number of pulses detected per 6 h of blood serial collection. ([b, c, d, e]) Values with different letters are significantly different ($P < 0.05$).

Table IV. Influence of passive immunization against testosterone on the amplitude of LH pulses (ng/ml)[a] in plasma of ovariectomized high fed heifers (H) and underfed heifers with E_2 (LE_2) or without E_2 (L).

Group of heifers	*Days from immunological treatment*					
	−1	*+1*	*+3*	*+8*	*+20*	*+30*
H ($n = 4$)	$0.5^b \pm 0.2$	$0.6^b \pm 0.3$	$0.8^b \pm 0.2$	$0.5^b \pm 0.1$	$0.6^b \pm 0.1$	$0.5^b \pm 0.2$
L ($n = 3$)	$1.3^b \pm 0.7$	$0.9^b \pm 0.8$	$0.8^b \pm 0.5$	$0.6^b \pm 0.1$	$0.4^b \pm 0.1$	$0.6^b \pm 0.1$
LE_2 ($n = 4$) $0.2^c \pm 0.02$		$0.2^c \pm 0.02$		$0.1^c \pm 0.02$		$0.2^c \pm 0.04$

[a] Mean ± SD. ($^{b, c}$) Values with different letters are significantly different ($P < 0.05$).

Before immunization, number of LH pulses was lower in the L than in the H group. These data agree with the observations of Imakawa *et al* (1987). This decrease was independent of ovarian steroids. The increase in amplitude of pulses of LH in the L group may have resulted from the decrease in pulse number as a longer period would have resulted between pulses allowing the pituitary to build up stores of LH. Nevertheless, pulsatility of LH in L group is similar to that found in the early luteal phase of cyclic cows (Rahe *et al,* 1980; Schallenberger and Walter, 1985). Thus the pituitary in heifers fed the diet limited in energy is able to secrete large amounts of LH. The pituitary of nutritionally induced anoestrous heifers has the ability to secrete LH in large amounts following gonadal removal (Imakawa *et al,* 1986).

E_2 implants produced physiological concentrations of E_2, in the underfed ovariectomized heifers, which were comparable with those of intact normal fed heifers prior to oestrus (Thibier and Saumande, 1975). Baseline, mean concentration and pulsatility of LH in LE_2 group were strongly depressed by E_2, which is in accordance with the results of Imakawa *et al* (1987).

The rise of LH pulsatility in L and LE_2 heifers after anti-testosterone immunoglobulin injection was consistent with results of other studies which have demonstrated that in ewes (Martensz *et al,* 1979; Martensz and Scaramuzzi, 1979; Webb *et al,* 1984; Thomas *et al,* 1987) and in cows (Campbell *et al,* 1985, Chang, 1987; Price *et al,* 1987; D'Occhio *et al,* 1988) immunized either against oestrogen, androgen or progestagen, the pituitary secreted more LH. These results demonstrate that high antibody specificity is not important to the physiological response, and support the idea that immunization against steroid results in a reduced concentration of biologically active steroid and decreased negative feedback leading to an increase in LH levels. The present findings of increased pulsatility of LH in immunized underfed heifers indicate that the testosterone antibodies could decrease negative feedback by cross-reacting with and neutralizing circulating E_2 in LE_2 group, or neutralizing non-ovarian steroids in the L group. In the L groups, these steroids may

come from a change in peripheral metabolism, and/or an increase of secretion by the adrenal. But most of the published works did not show any significant change of plasma cortisol after a long-term restriction in energy or protein in cattle (Anderson *et al,* 1988; Schrick *et al,* 1990). Moreover bovine adrenal can produce a lot of steroids (Dorfman and Ungar, 1965) and qualitative changes in adrenal secretion are possible.

In conclusion, passive immunization against testosterone increased sex steroid-binding levels in peripheral plasma and presumably reduced the amount of steroids (ovarian or non-ovarian) available for negative feedback effect on LH secretion. Thus the decrease of SBP binding capacity may contribute to the increase of steroid negative feedback on LH secretion in underfed cattle.

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