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Original article

Increase of plasma eCG binding rate after administration of repeated high dose of eCG to cows

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Abstract — Equine chorionic gonadotrophin (eCG) is still used to promote follicular growth in cattle and, more recently with an increased frequency of administration, in ovum pick-up protocols. The aim of this experiment was to verify the possible effect of high frequency of administration on the immune response to eCG. The profiles of eCG binding rate, in the blood of two groups (A, B) of 4 primiparous cross breed beef cows (3–3.5 years old) submitted weekly for 5 to 10 weeks to repeated high doses (1000–2000 IU) of equine chorionic gonadotrophin, are presented in this paper. A sensitive radiometric method was used to detect antibodies in plasma. The profiles clearly indicated a marked increase of eCG binding rate after 3 to 5 injections of the exogenous hormone to the females. The statistical analysis of the results established that treatments induced a significant increase ($P < 0.01$) in binding rates after 6 and 3 injections in group A and B respectively. These binding rates remained elevated for at least 1 week following the last injection and decreased afterwards. The values of plasma binding rates following repeated eCG administration differed significantly between groups (0.90 ± 1.04 and 1.04 ± 0.11 for groups A and B before treatment versus 11.77 ± 0.92, 6.70 ± 0.85 for groups A and B after treatment, $P < 0.01$) and from one cow to another ($P < 0.01$) with some cows presenting no significant immune response while others were more reactive against the hormone (at least 3 injections).

cow / eCG / antibody

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Résumé — Augmentation des taux de liaison plasmatique à l'eCG chez des vaches soumises à des administrations répétées de fortes doses de l'hormone. La gonadotrophine chorionique équine (eCG) est encore utilisée actuellement dans de nombreux protocoles de stimulation ovarienne chez les bovins, dont la superovulation et la ponction échoguidée, et, dans ce dernier cas, à intervalles très rapprochés. En vue de tester l'effet de ces hautes fréquences d'administration sur la réaction immune à l'eCG, huit vaches croisées viandeuses primipares de 3 à 3,5 ans ont reçu, de façon répétitive et à intervalles de 7 jours, des doses élevées (1000–2000 IU) de l'hormone et ce durant 5 à 10 semaines. La détermination radio-immunologique des taux de liaison plasmatique à l'eCG a été réalisée pour chacune de ces femelles. L'observation des profils indique une nette augmentation des taux de liaison après 3 (groupe B) à 5 injections (groupe A) de l'hormone aux femelles. Le traitement a eu une influence significative ($P < 0,01$) respectivement après 6 (A) et 3 (B) injections sur les taux de liaison plasmatique qui, par ailleurs, sont restés élevés au minimum 1 semaine après la dernière injection avant de décroître. La réponse aux traitements en terme de taux de liaison mesurés a été significativement différente entre groupes (0,90 ± 1,04 et 1,04 ± 0,11 pour A et B avant traitement, vs. 11,77 ± 0,92 et 6,70 ± 0,85 pour A et B après traitement, $P < 0,01$) et d'une femelle à l'autre ($P < 0,01$), certaines ne présentant pas de réponse significative au traitement tandis que d'autres étaient plus réactives (dès la 3^{e} injection).

vache / eCG / anticorps

1. INTRODUCTION

Equine chorionic gonadotrophin (eCG), also called PMSG (Pregnant Mare Serum Gonadotrophin), is specific to the mare. It is a glycoprotein that displays both LH- and FSH-like activities when used in other species than the horse. Equine CG is synthesized by the endometrial cups of the equine placenta between day 40 and day 130 of pregnancy [1]. Similarly to the hCG produced in the syncytiotrophoblast of pregnant woman, it contains higher amounts of carbohydrate side chains and sialic acid when compared to the pituitary gonadotrophins. The molecular weight of eCG was estimated at about 45 kDa with 47% of the molecule consisting of carbohydrates [14]. The plasma half-life of eCG was determined to be about 6 days in horses [10] and 5 to 15 days in cattle [26] and is mainly due to the terminal sialic acid residues of its N- and O-linked saccharide chains [25, 37]. These biochemical characteristics and its availability in large quantities and at a low cost made eCG a molecule frequently used to promote follicular growth and to control ovulation in cattle.

Both pituitary and placental gonadotrophins are composed of α- and β-subunits non-covalently associated [27]. The α-subunit of eCG is comprised of a peptide chain of 96 amino acids and 2 oligosaccharide chains. The β-subunit contains 149 amino acids [14, 27]. Therefore, because of its heterologous origin, its molecular structure, its high molecular weight and its high level of glycosylation, eCG appears to be potentially immunogenic when used in cattle.

The first descriptions of refractoriness to repeated gonadotrophic treatments were reported in the 1930's: cows [20], sheep [23], human [41]. In 1953, Willet [40] repeatedly treated cows with gonadotrophins to induce the development of refractoriness to treatments: (a) five daily subcutaneous injections of sheep or hog FSH – 30 to 50 Gram-equivalents of desiccated pituitary – followed on the 6th day by an i.v. injection of unfractionated sheep pituitary gonadotrophin; or (b) one subcutaneous injection of eCG – 2000, 3000, 5000 or 10000 IU – followed 5 days later by an i.v. injection of hCG – 5000 IU – or unfractionated sheep gonadotrophin. Studying the same females, he showed that long periods without

treatments were ineffective in overcoming refractoriness and that increased dosages could partially but only temporarily restore some degree of responsiveness.

Jainudeen [22] hypothesised that the refractoriness against exogenous gonadotrophins could be mediated by antibodies. In 1979, Roser [30] confirmed the existence of anti-gonadotrophins antibodies in the blood of mares repeatedly treated with 2600 IU of hCG during each oestrus for 5 or 6 consecutive oestrous cycles.

In cattle, eCG is largely used in zootechnical programs in order to stimulate follicular growth and ovulation: treatment of anoestrus [19], synchronisation or induction of oestrus in combination with progestagens (400 to 600 IU once a year) [7, 12], induction of superovulation (1000 to 2000 IU) [2, 16, 35] and more recently, stimulation of follicular growth in ovum pick-up programs (OPU) [15, 21]. The higher doses and the higher frequency administration of eCG in OPU programs may have an influence on eCG antibody production in the cow and may induce a reduction in the efficiency of such treatments. In goats and ewes, estrus induction/synchronisation and superovulation constitute the most common use of gonadotrophins. Equine chorionic gonadotrophin (250 to 400 IU) is frequently associated with progestagens which are administered for a period similar to the lifetime of a cyclic corpus luteum. Baril et al. [6] showed that the eCG binding rate before treatment increased with the number of treatments the goats had received previously and led to an increase in the frequency of late estrus. When eCG binding was higher than 10%, the percentage of late estrus was high and the fertility after AI at a predetermined time decreased significantly. The use of eCG was also reported in OPU protocols in sheep [36].

The purpose of the present study was to investigate the expression of an immunological response of cows submitted to repeated injections of high doses (1000 to 2000 IU) of eCG for 5 and 10 weeks.

2. MATERIALS AND METHODS

2.1. Animals and treatment

Two groups (A and B) of 4 primiparous cross breed beef cows between 3 and 3.5 years old were used for this experiment.

Cows of the group A received 10 injections of eCG (i.m., once a week, Folligon; Intervet, France) (2000 IU; 4×1000 IU; 5×2000 IU) while cows of the group B received 5 eCG treatments (2000 IU each week).

All the cows were sampled before receiving any eCG treatment. During the period of treatment, all the cows were sampled three times a week. Blood samples were also collected over a period of three weeks following the end of eCG administration.

2.2. Assay

2.2.1. Radiolabeled tracer (equine Chorionic Gonadotrophin)

A pure preparation of eCG was used for the radiolabeling. Equine chorionic gonadotrophin was purified according to Christakos and Bahl [11].

2.2.2. Radiolabeling with ^{125}Iodine

The purified eCG was radioiodinated with ^{125}I (Amersham IMS-30, Gent, Belgium) according to the enzymatic procedure of Thorell and Johansson [38]. In order to obtain a high specific radioactivity and a reliable tracer, 12.5 μg of eCG (dry weight lyophilized powder) were iodinated using 1 milliCurie of ^{125}I, 1 μg of lactoperoxydase (Boehringer Mannheim, Mannheim, Germany) and 20 μL of H_2O_2 (Perhydrol Merck 1/30,000, Darmstadt, Germany). Immediately after the reaction (4 min), the radiolabeled hormone was separated from free ^{125}Iodine by chromatography on a Sephadex (Pharmacia, Uppsala, Sweden)

G-75 column (0.9 × 30 cm) equilibrated with bSA-Tris buffer (Tris HCl 25 mM, $MgCl_2$ 10 mM, pH 7.6, bSA 0.1%).

2.2.3. Antibody detection in plasma samples

Blood was collected at the jugular vein, incubated at 20 °C during 24 hours, and centrifuged (20 min at 1500 *g*). Sera were frozen and stored at –20 °C until assay.

All dilutions of sera or tracer were performed in Tris-bSA buffer containing bSA 0.1% and Neomycin sulfate 0.01%. The incubation volume was always 500 µL.

Reagents were added in the following order.

– 300 µL of Tris-bSA buffer;

– 100 µL of each serum diluted 10 times in Tris-bSA buffer;

– 100 µL of ^{125}I-eCG corresponding to 10000 cpm (T) or to 1.4 ng of hormone (as determined by the self-displacement method of Roulston [31]).

Incubation was carried out for 16 h at 20 °C. Thereafter, 100 µL of a donkey anti-cow immunoglobulin solution were added. Incubation was carried out for 1 additional hour at 20 °C. Then 500 µL of 4% (w/v) polyethylene glycol (PEG MW 10000 Merck Inc., Darmstadt, Germany) diluted in Tris-bSA buffer were added. The tubes were centrifuged at 2500 *g* for 20 min and the supernatants aspirated.

The pellets were washed with 3 ml of Tris-bSA buffer and centrifuged at 2500 *g* for 15 min. After aspiration of the supernatants, the pellets were counted for radioactivity in a gamma counter with a 60% efficiency for ^{125}I.

Non specific binding (NSB) of ^{125}I-eCG was determined using plasma (in duplicate) obtained from the cows before any administration of the exogenous gonadotrophin.

2.3. Evaluation of results

The data are presented as eCG binding rates (B.R.) which represent the capacity for the serum to bind ^{125}I-eCG used in the assay.

2.4. Statistical analysis

Statistical analysis of results was made using the SAS General Linear Model Procedure (GLM) [34] with binding rate considered as the dependent variable and animals and treatments as independent variables. For the statistical analysis, groups were considered separately and the experiment was divided into three periods according to the observed B.R. profiles: day 0 to day 4 (before treatment), day 5 to day 32 (intermediary period) and after day 32 (effective B.R. increase). The influence of the number of eCG injections was also tested in each group. Finally, the injections were tested for the "booster" effect they could have on eCG B.R., i.e. we tested the influence of an injection on the response the following one induced in terms of B.R. increase.

3. RESULTS

3.1. Effect of the treatment on eCG plasma binding rates

The non-specific binding of the sera to the radiolabeled tracer (^{125}I-eCG) before treatment did not differ significantly between the two groups of cows (mean = 0.90 and 1.04% respectively for group A and B). Repeated injections of eCG to cows were followed by a variable response ($P < 0.01$) in terms of plasma eCG binding with some of the cows presenting an increase in eCG binding rates with repetition of treatments (Figs. 1 and 2). A significant difference ($P < 0.01$) was found for B.R. levels before treatment and after the 32nd day, in both groups

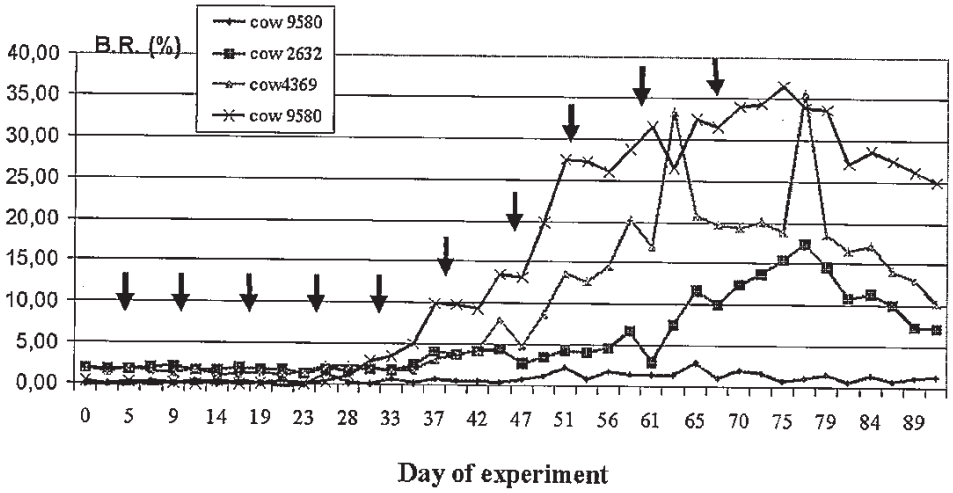

Figure 1. eCG binding rate follow-up in group A. Each cow separately. Arrows indicate eCG injections.

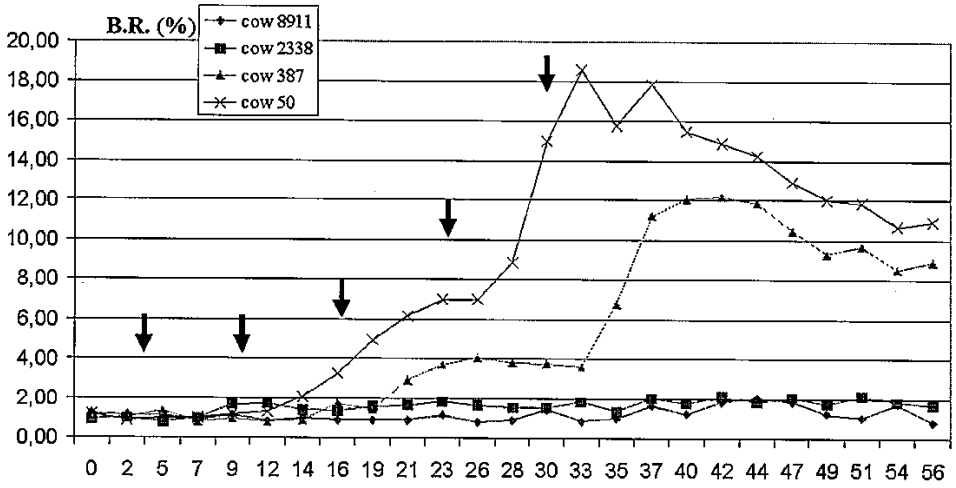

Figure 2. eCG binding rate follow-up in group B. Each cow separately. Arrows indicate eCG injections.

(0.90 ± 1.04 and 1.04 ± 0.11 for groups A and B before treatment vs. 11.77 ± 0.92, 6.70 ± 0.85 for groups A and B after treatment, $P < 0.01$) and for B.R. between period 2 (day 5–day 32) and 3 (> day 32) ($P < 0.01$) but the difference was not significant between period 1 (day 0 to day 4) and 2 (day 5 to day 32) ($P \geq 0.05$) (Tab. I). The binding rates remained elevated or continued to increase for at least 1 week following the last injection and decreased afterwards.

3.2. Cows of group A

Three cows (2632, 4736, 4369) presented a marked increase in B.R. levels after the 5th injection (Fig. 1). The highest values

Table I. Respective effects of protocol (type of treatment) and treatment on eCG binding rate (*P* value) when comparing transition between determined periods of the experiment.

Effects on B.R.	1st period (0 d–5 d) compared with 3rd period (33 d...)	1st period (0 d–5 d) compared with 2nd period (6 d–32 d)	2nd period (6 d–32 d) compared with 3rd period (33 d...)
Protocol	0.31	0.14	0.07
Treatment	0.00097*	0.14	6.09×10^{-12}*
Protocol-Treatment	0.29	0.22	0.0022*

* Significant at 1% level.

for eCG binding were obtained with this protocol after the last injection. Cow 9580 remained with a low level of B.R. during the three months of the experiment.

3.3. Cows of group B

The maximum value for eCG binding was reached in cow 50 just after the last administration of the hormone. Equine CG binding remained at basal level in cows 8911 and 2338 and increased after the third injection in cows 50 and 387 (Fig. 2).

3.4. Effect of the number of treatments

In group A, a significant effect of the hormone administration on plasma B.R. was established after 6 injections ($P = 0.01$). Each following treatment induced a significant increase in B.R. ($P = 0.02$; 0.007; 0.004; 0.0008 respectively for treatments 7, 8, 9 and 10). This effect was significant after 3 injections in group B ($P = 0.02$) and remained significant for the following injections ($P = 0.05$; 0.007 respectively for treatments 4 and 5).

No significant influence of an injection on the response the following one induced was found, except for transition from the 3rd to the 4th injection in group A ($P = 0.04$) and from the 2nd to the 3rd injection in group B ($P = 0.03$).

4. DISCUSSION

Our study is the first in which the plasma eCG binding rate was estimated in cows which had received such a high dose and frequency of injection. The use of eCG in order to induce estrous synchronisation was previously restricted to one administration per year [16, 18]. In superovulation protocols the frequency of injection was increased to one injection every two months and in ovum pick up protocols the frequency was highest with an injection weekly or twice a month.

The problem of active immunisation of the females after repeated treatments constitutes a major drawback in long-term reproductive programs. In a study with cows receiving 3000 IU of eCG at intervals of six months, Jainudeen [22] observed no differences in ovulatory response when comparing the response to either the first or the second treatment. When this dose was repeated at intervals ranging from 31 to 40 days or 18 to 21 days respectively, the same author found that eCG failed to stimulate the ovaries.

The cows of our experiment were treated once a week. The objective of using a regimen with such high concentrations of eCG and high frequency of injections was to test for a rapid increase in anti-eCG antibodies as high dose and high frequency protocols are used in OPU programs. This investigation on plasma binding rates to eCG showed a

clear immunological response in 5 cows of the 8. This immunological response can be explained by the long half-life of the eCG [17] and the short interval (one week) between injections that did not allow a complete clearance of the molecule from the circulation and allowed a possible cumulative effect of the dose injected.

The delay of 3 to 5 weeks for the cows to elicit an immune response is in good agreement with the time needed for an immune response following antigen injection as reported in the literature. The period necessary for the production of antibodies generally varies from 24 h to 2 weeks and is related to the nature and the dose of the antigen as well as the adjuvant and route of injection [3].

We observed an important variability of the immune response to eCG in both our protocols and this variability has also been described in other species: Bourdillon and Lebas in rabbits [9, 24], Remy in goats [28], Roser in horses [30] showed that some females presented a high response when others gave very low responses. This variability could be dependent on the genotype of the females [32, 33]. In goats, Baril [4, 5] suggested the existence of different populations of females, classified as low, medium and high responders to eCG administration. The demonstration by Roy [32, 33] of the correlation between the major histocompatibility complex type and the individual variability of the humoral immune response of goats to eCG, confirmed that the high or low plasma antibody concentration was an inherent and repeatable characteristic of each individual [32]. In our study, this variability of the response to treatment was confirmed by the fact that the effects of the treatments were significantly different from one cow to another, either in group A or in group B.

The maximum value in plasma B.R. was reached after the last administration of the hormone to the females in both protocols. Roser [30] showed in the horse that plasma anti-gonadotrophin antibodies increased after 2 to 5 treatments with hCG. In the goat, Remy [28], Baril [5] and Gonzalez [18] also reported a large variability. This phenomenon is not in contradiction with the long half life of the hormone [10] nor with the temporary persistence of the stimulation of antibodies production. A progressive decrease in plasma gonadotrophin binding rates was observed with the end of the treatments, as described in other studies [6, 8, 28, 30].

This study allowed us to investigate, by a short-term protocol, the immune response that might occur in long-term protocols. This side effect is frequently not taken into account or not clearly revealed in short-time studies, in farm programs or in ovum pick-up protocols. The use of the homologous hormone of the treated species is probably the solution to the specific problem of antibody production [35]. Other solutions to improve reproductive efficiency, without inducing refractoriness, could be cited: superactive analogues of recombinant hTSH have been recently developed, with increased receptor binding affinity, bioactivity and plasma half-life, in order to avoid the development of neutralizing and hemagglutinating antibodies [29, 39] after repeated administration of TSH in humans. Also site-specific mutagenesis of immunodominant epitopes of proteinaceous drugs such as staphylokinase [13] allowed reduction of their immunogenicity. This engineering of proteins could be applied to the reproductive glycoproteins to produce a new generation of stimulatory agents of the ovary presenting a high ratio of activity to antigenicity.

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