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Delboni Stuhr, Bhárbara; Santos Gil, Isaac; Ferreira Lacerda, Tracy; Gomes Favoreto, Mauricio; Loureiro, Bárbara

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Consequences of eight consecutive applications of equine chorionic gonadotropin on ovarian follicle development and ovulation in *bos indicus* cows

Consequências do uso consecutivo de oito doses de gonadotrofina coriônica equina no desenvolvimento folicular e ovulação de vacas *bos indicus*

Bhárbára Delboni Stühr¹ Isaac Santos Gil¹ Tracy Ferreira Lacerda¹
Maurício Gomes Favoreto¹ Bárbara Loureiro^{1*}

ABSTRACT

The equine chorionic gonadotropin (eCG) is a glycoprotein produced in mare endometrial calices. In bovine, it is used in estrus synchronization protocols. However, studies have shown that it is potentially immunogenic and its effect can decrease after repetitive use. This study aimed to evaluate antral follicle dynamics, corpus luteum (CL) and ovulation rate in *bos indicus* cows submitted to an estrus synchronization protocol in association with eCG for eight times consecutively. Ten cyclical, multiparous, and pasture raised beef cows were divided into two groups: control group (n=5) and eCG group (n=5). In 30 day interval, all animals were synchronized with the same estrogen/progesterone based protocol, totalizing 8 re-synchronizations. Cows in the treatment group received 300IU eCG 48 hours prior to the presumable ovulation. Ultrasonnd examinations were performed on Day 4 of the protocol (approximately 1.5 days after follicle recruitment) to count antral follicles, on Day 10, to count antral follicles and to measure size of the largest follicle and on Day 18 to measure the diameter of the CL. No difference ($P>0.05$) between follicular growth and size of the pre-ovulatory follicle was reported between groups. Cows treated with eCG had a larger ($P<0.05$) CL and increased ($P<0.05$) ovulation rate (18mm and 92%, respectively) when compared with control group (14.1mm and 80%, respectively). Furthermore, consecutive treatments did not affect CL nor ovulation rates. In conclusion, eCG treatment increased CL size and ovulation rate even after 8 consecutive treatments.

Key words: corpus luteum, eCG, fertility, immunity, FTAI.

RESUMO

A gonadotrofina coriônica equina (ECG) é uma glicoproteína produzida nos cálices endometriais da égua. Em bovinos, é utilizada em protocolos de sincronização do estro. Estudos têm mostrado que ela é potencialmente imunogênica e seu efeito pode diminuir depois de utilização repetida. O objetivo do presente trabalho foi avaliar a dinâmica folicular, o

diâmetro do corpo lúteo (CL) e taxa de ovulação em vacas *bos indicus* submetidas a um protocolo de sincronização estral com eCG por oito vezes. Dez vacas de corte, cíclicas, multíparas e solteiras foram divididas em dois grupos: grupo controle (n=5) e grupo eCG (n=5). Em um intervalo de 30 dias, os animais foram sincronizados com um protocolo de estrógeno/progesterona, totalizando 8 re-sincronizações. Os animais do grupo tratamento receberam 300UI eCG 48 horas antes da provável ovulação. Exames ultrasonográficos foram realizados no dia 4 do protocolo (aproximadamente 1,5 dias após o recrutamento folicular), para contar foliculos antrais, no dia 10, contar foliculos antrais e medir o tamanho do maior folículo e, no dia 18, para medir o diâmetro do CL. Não foi encontrada diferença ($P>0,05$) entre foliculos no dia 4 ou 10. Vacas tratadas com eCG tinham um maior ($P<0,05$) CL e elevada ($P<0,05$) taxa de ovulação (18mm e 92%, respectivamente) quando comparadas ao grupo controle (14,1mm e 80%, respectivamente). Além disso, tratamentos consecutivos não afetaram o CL nem as taxas de ovulação. Em conclusão, o tratamento com eCG aumentou o tamanho do CL e taxa de ovulação, mesmo depois de 8 tratamentos consecutivos.

Palavras-chave: corpo lúteo, fertilidade, imunidade, IATF.

INTRODUCTION

Equine chorionic gonadotropin (eCG) is a glycoprotein hormone molecule (ROY et al., 1999) produced in the endometrial calices of pregnant mares between 40 to 130 days of gestation (CATCHPOLE & LYONS, 1934). When applied exogenously, the eCG creates conditions for follicular growth and ovulation by binding FSH and LH follicle receptors and LH receptors of the corpus luteum (BARUSELLI et al., 2004).

¹Laboratório de Fisiologia da Reprodução, Universidade Vila Velha (UVV), Av. Comissário José Dantas de Melo, 21, 29102-020, Vila Velha, ES, Brasil. E-mail: barbara.loureiro@uvv.br. *Corresponding author.

Association of eCG with hormonal estrus synchronization protocols can increase ovulation and pregnancy rates, especially in post-partum and low body condition score (BCS) cows. In these animals, eCG induces ovulation of the largest and most functional follicle, which therefore will develop a better corpus luteum (CL), increasing progesterone, which favors embryonic development and survival (RENSIS & LÓPEZ-GATIUS, 2014).

The eCG should also be used in lactating females, as it eliminates temporary calf removal, which when held for only 48 hours, causes stress on the mother and calf (SILVEIRA et al., 2010). Conversely, eCG was not beneficial when used in Holstein and post-partum Nellore cows that were in a good BCS (ERENO et al., 2007; PULLEY et al., 2013).

However, due to a heterologous origin, a high molecular weight, high degree of glycosylation and longer half-life, eCG becomes a potentially immunogenic molecule when injected in cattle (DRION et al., 2001). Thus, animals that undergo successive treatments with eCG appear to become refractory to this molecule, not effectively responding to the treatment (DRION et al., 2001).

The production of anti-eCG antibodies has been reported in goats, sheep, cows, and rabbits (ROY et al., 1999a; ROY et al., 1999b; DRION et al., 2001; THEAU-CLEMENT et al., 2008, respectively). These antibodies are responsible for reducing the interaction of eCG with FSH and LH receptors (MAUREL et al., 2003). As a consequence a delay could occur in the LH surge and ovulation, reducing the females' fertility. Some studies report antibody production from the first eCG application while others claim that there is an additive effect in repeated applications (ROY et al., 1999a; DRION et al., 2001; MANTOVANI, 2010).

Thus, this study aimed to evaluate follicular growth, diameter of the pre-ovulatory follicle and CL, as well as ovulation rate in *bos indicus* cows submitted to an estrous synchronization protocol associated with eCG for eight consecutive times.

MATERIALS AND METHODS

The experiment was performed from January to September 2015 at a farm located in Linhares, ES (19°11'4''S and 39°49'35''W). Ten crossbred *bos indicus* cows, aged between 4 and 6 years old, with BCS ranging from 3 and 3.5 (scale of 1-5; LOWMAN et al., 1976), multiparous and non suckled, were used. The animals were pasture raised (*Brachiaria humidicola*), receiving mineral

salt and water *ad libitum*. All ten animals were from the same commercial farm and had been submitted to two estrus synchronization protocols with eCG (200 IU; Novormon; Zoetis, São Paulo, SP, Brazil) in the previous three reproductive breeding seasons. All the cows became pregnant in each season and had the same parity number.

The animals were divided into two groups: control (n=5) and eCG (n=5). The entire hormonal treatment was done in all the animals in each protocol, being repeated 8 times with a 30 day interval. The hormonal protocol and ultrasound scheme are shown in figure 1.

Briefly, all females received, on a random day of the estrous cycle, 2 mg of estradiol benzoate intramuscularly (Sincrodiol, Ouro Fino, São Paulo, SP, Brazil) and an intravaginal progesterone implant (PRIMER; 1g of progesterone; Tecnopec, Guaçu, SP, Brazil), being considered as Day 0 (Day 0).

On Day 4 of the hormonal treatment (approximately 1.5 days of follicular recruitment; SÁ-FILHO et al., 2011) animals were evaluated by ultrasound (US; MINDRAY, DP-2200VET model with 7.5MHz linear transducer) for antral follicle count and presence and location of preexisting corpus luteum (CL) prior to hormonal treatment.

On Day 7, 500mg of sodium cloprostenol (Ciosin; MSD Saúde Animal, Cotia, SP, Brazil) was

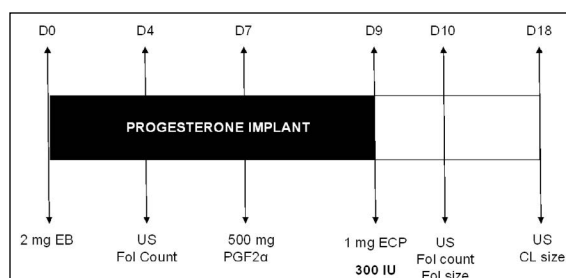


Figure 1 - Estrus synchronization protocol and ultrasound (US) scheme performed in *bos indicus* cows from control (without eCG) and treatment (eCG) groups. On Day 0 (D0; an aleatory day of estrus cycle) cows received 2 mg of estradiol benzoate and a progesterone vaginal implant was inserted, on D4 of the protocol (approximately 1.5 days after follicle recruitment) antral follicles (fol) were counted and on D7 cows received and injection of PGF2α. On D9 the implant was removed and cows received 1 mg of estradiol cypionate, cows from the treatment group also received 300IU of eCG. On D10 an ultrasound examination was performed to count the number of follicles and measure the diameter of the largest follicle. On D18 another ultrasound examination confirmed the presence and measured the diameter of the corpus luteum (CL).

administered intramuscularly. After 48 hours, on Day 9, the intravaginal implant was removed and 1mg of estradiol cypionate applied intramuscularly (ECP; Zoetis, São Paulo, SP, Brazil). Females belonging to the eCG group also received in this time period 300 IU of ECG intramuscularly (Novormon; Zoetis, São Paulo, SP, Brazil). All handling was performed at the same time (0800).

On Day 10 the amount of antral follicles and diameter size of the largest follicle in the right and left ovary was evaluated using an US. On Day 18 (seven days after presumable ovulation) an ultrasound evaluation for presence and diameter of the corpus luteum in response to the hormonal treatment was performed.

Statistical analysis

The effect of treatment (control or eCG), protocol number and its interaction on the number of follicles on Day 4 and Day 10, follicle size at Day 10 and CL size on Day 18 in the right or left ovaries was analyzed by analysis of variance using the PROC MIXED procedure of SAS (SAS for Windows, Version 9.0, Cary, NC), with the animal (cow) being considered the subject and protocol number the repeated measure. Individual comparisons were performed using the PDIF function (probability of individual differences). Percentage of animals ovulating per protocol was assessed by analysis of variance using the PROC GLM procedure of SAS; data were transformed to arcsine before analysis. Results are presented as least square means (LSmeans) and standard error of the mean (SEM).

RESULTS

Number of follicles on Day 4 and Day 10, follicle size on Day 10 and CL size on Day 18 as well

as ovulation rate per treatment (control or eCG) per protocol number (1 to 8) are presented in table 1.

No difference was observed in the number of antral follicles on Day 4 between control and eCG treated animals nor an interaction between protocol number and treatments ($P>0.05$; Figure 2A). Also, no difference was observed in the number of follicles ($P>0.05$; Figure 2B) or size of the largest follicle on Day 10 (Figure 2C) between control or eCG treated animals nor an interaction between protocol number and treatment ($P>0.05$).

Cows treated with eCG had a larger ($P=0.04$) CL on Day 18 when compared with cows from the control group (Figure 2D). Furthermore, ovulation rate was higher ($P=0.03$) on eCG treated cows when compared with control cows (92 ± 0.3 vs. 80 ± 0.3). Additionally, the administration of eight doses of eCG consecutively did not alter ovulation rate per protocol ($P>0.05$).

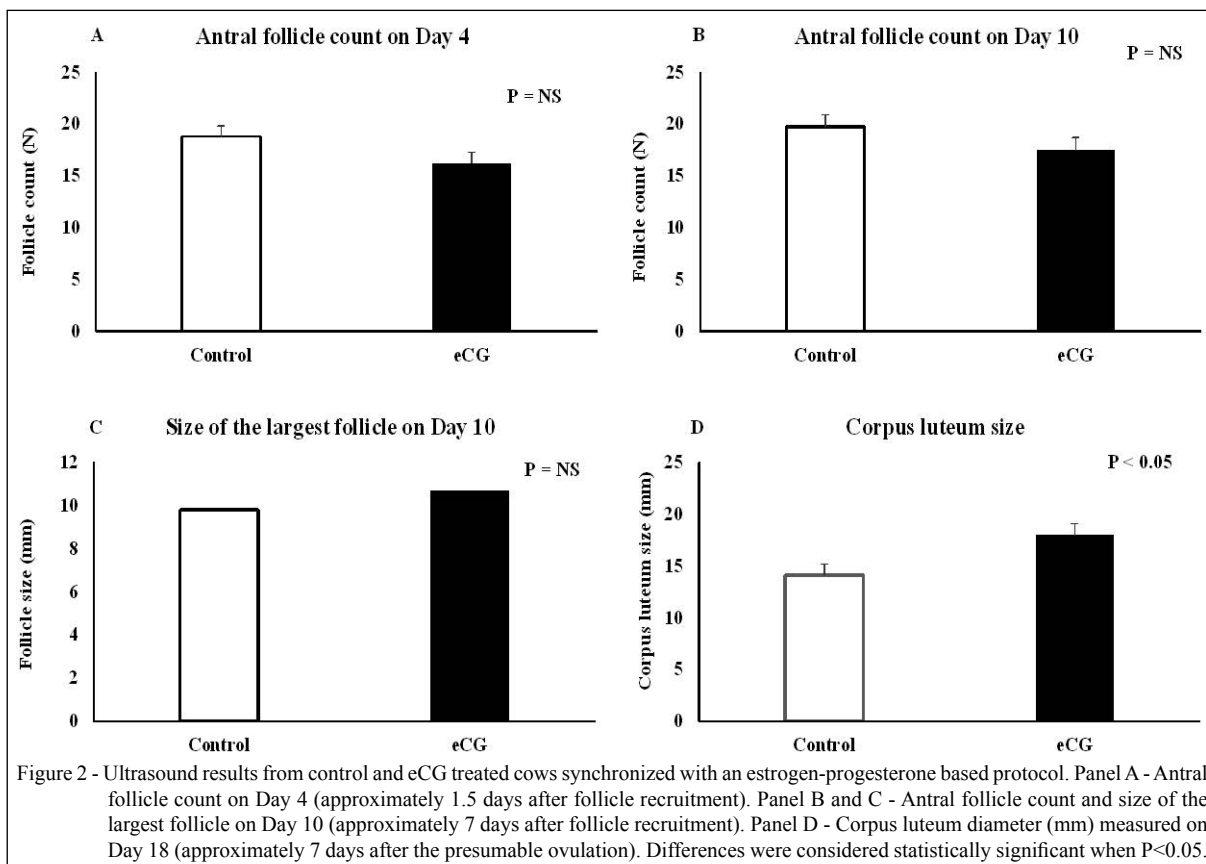
DISCUSSION

The use of eCG for eight times consecutively did not decrease ovulation as hypothesized previously or influence the follicular wave of the subsequent estrus cycle. Furthermore, animals in the eCG group had a larger CL, but no difference was observed in the number of follicles or the pre-ovulatory follicle size after eCG administration.

The eCG is usually used in estrus synchronization protocols as a follicle developing agent due to its FSH- and LH-like activity (TORTORELLA et al., 2013). However, some authors have suggested that due to the protein's size and long plasma half-life an immune response against the eCG molecule can be developed by the animals which would decrease the

Table 1 - Number of follicles (Num Fol) on Day 4 (D4) and Day 10 (D10), follicle size on day 10 (D10), corpus luteum (CL) size on day 18 (D18) and ovulation rate of *bos indicus* cows submitted to an estrus synchronization protocol without (control) or with 300IU of equine chorionic gonadotrophin (eCG). Results are LS means \pm standard error.

Protocol Sequence	-----Fol D4 (n)-----		-----Fol D10 (n)-----		---Fol size D10 (mm)---		---CL size D18 (mm)---		---Ovulation rate (%)---	
	Control	eCG	Control	eCG	Control	eCG	Control	eCG	Control	eCG
1 st	20 \pm 2.85	21.6 \pm 2.85	22.4 \pm 3.5	20.6 \pm 3.5	10.728 \pm 1.7	11.312 \pm 1.7	18.52 \pm 3.3	17.66 \pm 3.3	80	80
2 nd	16.8 \pm 2.85	19.4 \pm 2.85	24.2 \pm 3.5	18.6 \pm 3.5	9.19 \pm 1.7	12.14 \pm 1.7	12.64 \pm 3.3	17.94 \pm 3.3	80	100
3 rd	18.6 \pm 2.85	16.8 \pm 2.85	20.2 \pm 3.5	16.4 \pm 3.5	9.32 \pm 1.7	11.33 \pm 1.7	12.78 \pm 3.3	15.1 \pm 3.3	80	80
4 th	19.4 \pm 2.85	17.2 \pm 2.85	20.4 \pm 3.5	22.2 \pm 3.5	9.91 \pm 1.7	8.85 \pm 1.7	14.26 \pm 3.3	12.14 \pm 3.3	80	80
5 th	16.6 \pm 2.85	12.8 \pm 2.85	16.6 \pm 3.5	13.4 \pm 3.5	9.27 \pm 1.7	10.76 \pm 1.7	13.22 \pm 3.3	22.78 \pm 3.3	80	100
6 th	23.4 \pm 2.85	13.4 \pm 2.85	16 \pm 3.5	12.4 \pm 3.5	11.56 \pm 1.7	9.54 \pm 1.7	10.28 \pm 3.3	19.26 \pm 3.3	60	100
7 th	17.8 \pm 2.85	15.4 \pm 2.85	23 \pm 3.5	16.6 \pm 3.5	10.44 \pm 1.7	11.04 \pm 1.7	16.58 \pm 3.3	18.36 \pm 3.3	100	100
8 th	17.8 \pm 2.85	13.4 \pm 2.85	14.8 \pm 3.5	20 \pm 3.5	8.50 \pm 1.7	11.19 \pm 1.7	14.98 \pm 3.3	21.4 \pm 3.3	100	100



eCG's effect after repetitive use (revised by DRION et al., 1998; ROY et al., 1999a; DRION et al., 2001).

The positive effect of eCG on follicle development has not been completely determined. Some authors have shown that it can increase pre-ovulatory follicle size and that would end up in a larger CL (TORTERELLA et al., 2013). The size of the CL is important for progesterone secretion and pregnancy maintenance (RIGOGGIO et al., 2013; MARTINEZ et al., 2014). However, in previous studies eCG did not influence pre-ovulatory follicle size however it rather enhanced CL size (BARUSELLI et al., 2004; PULLEY et al., 2013; MARQUEZINI et al., 2014). Even though it was not observed an increase in pre-ovulatory follicle size it was observed a larger CL. An increase in progesterone concentration has also been described as a positive effect of the eCG (SANTOS et al., 2001; BARUSELLI et al., 2004; MARQUEZINI et al., 2014). The eCG acts increasing the number of luteal cells and mitochondrias that in turn increases pregnenolone synthesis (RIGOGGIO et al., 2013).

The use of eCG is more beneficial when animals are post-partum, lactating or in a low BCS (SILVEIRA et al., 2010; RENSIS & LÓPEZ-GATIUS,

2014). In this study single non-lactating cows with good BCS were used to test the negative effect of repetitive use of eCG. The above categories (post-partum, lactating or in a low BCS) were avoided so no other factor would negatively influence antral follicle development, ovulation rate and CL formation. We believed that in any of these situations the result (no detrimental effect of consecutive eCG use) would be the same.

The eCG administration can increase the concentration of anti-eCG antibodies after the first use (ROY et al., 1999b; MANTOVANI, 2010) and some studies have reported an additive effect of subsequent treatments (ROY et al., 1999a; DRION et al., 2001). Furthermore, most studies have concluded that there is an animal individuality to the immune response against eCG (DRION et al., 2001; MANTOVANI, 2010). This immune response can be especially deleterious to fertility. The anti-eCG antibodies can delay the LH surge and in consequence ovulation will be lated desynchronizing with the moment of FTAI (ROY et al., 1999a; MAUREL et al., 2003). Furthermore, female goats with high anti-eCG concentration showed low fertility after FTAI due to a low follicle development and decreased

steroid genesis (ROY et al., 1999a). MANTOVANI (2010) observed a negative correlation between the concentration of anti-eCG antibodies and number of antral follicles (>6mm) after the second treatment with 2000IU eCG. However, when a high dose of eCG (800IU) was administered before follicle deviation it increase double ovulation rate (MARTINEZ et al., 2014).

However, most of the studies that show an immunogenic action of eCG were performed using high doses of the hormone (500 IU in goats and sheep and 2000 IU in cows; ROY et al., 1999a; ROY et al., 1999b; DRION et al., 2001, respectively). Here, it was used 300 IU, dose that is routinely used in fixed time artificial insemination (FTAI) programs in *bos indicus* cows. Treatments were repeated eight times in a 30 day interval. Usually, a *bos indicus* cow will be hormonally stimulated one or two times per breeding season and will have approximately eight months of interval within seasons. It has been shown that a longer interval within injections will decrease eCG immunogenicity and increase immunity response time (ROY et al., 1999).

Here we showed that even after the eighth treatment the cows in the eCG group continued to ovulate. Furthermore, cows in the eCG group had a larger CL which is associated with better pregnancy rates and lower pregnancy losses (NOGUEIRA et al., 2012; GONELLA-DIAZA et al., 2013). It is believed that these results can be extrapolated to the field and in the dose of 300IU eCG can be repeatedly used in subsequent breeding seasons without any deleterious effect on fertility.

CONCLUSION

The repeated use of eCG, in the conditions of the present study, did not decrease antral follicle growth or ovulation rate. Contrariwise, the use of eCG in the FTAI protocol increased CL size.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The experiment was approved and followed the guidelines of the Ethics Committee for Animal Use of the Universidade de Vila Velha and the Sociedade Brasileira de Ciência em Animais de Laboratório (CONCEA), under the registration number 349/2015.

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