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Ovarian function, steroid hormones and fertility in cows stimulated with gonadotropins

Alexandre Barbieri Prata

Thesis presented to obtain the degree of Doctor in Science. Area: Animal Science and Pastures

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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SUMMARY

RESUMO 8
ABSTRACT 9
1. INTRODUCTION 11
References 12
2. EQUINE CHORIONIC GONADOTROPIN INCREASES FERTILITY OF GRAZING DAIRY COWS THAT RECEIVE FIXED-TIME ARTIFICIAL INSEMINATION IN THE EARLY BUT NOT LATER POSTPARTUM PERIOD 17
Abstract 17
2.1 Introduction 17
2.2 Material and methods 19
2.3 Results 21
2.4 Discussion 23
2.5 Conclusion 25
References 25
3. EFFECT OF DIFFERENT CHORIONIC GONADOTROPINS ON FINAL GROWTH OF THE DOMINANT FOLLICLE IN *BOS INDICUS* COWS 29
Abstract 29
3.1 Introduction 30
3.2 Material and methods 31
3.3 Results 33
3.4 Discussion 34
3.5 Conclusion 36
References 36
4. EFFECT OF GONADOTROPINS ON DEVELOPMENT OF THE OVULATORY FOLLICLE AND RESULTING CORPUS LUTEUM IN COWS 41
Abstract 41
4.1 Introduction 42
4.2 Material and methods 44
4.3 Results 47
4.4 Discussion 51
4.5 Conclusion 53

References 54
5. FINAL CONSIDERATIONS 61

RESUMO

Função ovariana, hormônios esteroides e fertilidade de vacas estimuladas com gonadotrofinas

Para aumentar a rentabilidade nos sistemas de produção de bovinos de corte e leiteiro, são necessárias estratégias de manejo reprodutivo que proporcionem elevadas taxas de serviço e concepção, especialmente no início da estação de monta em bovinos de corte e após o período de espera voluntária para rebanhos leiteiros. Para atingir esses objetivos, vários protocolos hormonais foram desenvolvidos com o intuito de sincronizar a onda folicular, o estro e a ovulação, permitindo assim inseminação artificial em tempo fixo (IATF). Considerando que a eCG é uma ferramenta indispensável para o manejo reprodutivo, é fundamental uma melhor compreensão de sua ação biológica no processo de crescimento folicular final, ovulação e desenvolvimento luteal, otimizando seu uso em protocolos hormonais. Além disso, alternativas para a eCG precisam ser testadas. Dessa forma é importante uma melhor compreensão de como FSH e LH atuam no folículo dominante. Com base nisso, três estudos foram realizados. O primeiro avaliou os efeitos da eCG na fertilidade de 679 vacas lactantes mestiças em sistema de pastejo sincronizadas para IATF. O tratamento com eCG tendeu em aumentar a P/IA aos 30 e 60 dias e aumentou a P/IA aos 30 e 60 dias para vacas inseminadas com DEL $\leq$ 70, mas não houve efeito nas vacas que receberam IA após 70 DEL. O segundo avaliou o efeito da eCG ou diferentes doses de hCG no crescimento final do folículo dominante em 84 vacas Nelore submetidas a IATF. Não houve diferença quanto ao diâmetro do maior folículo no D8 ou D10. No entanto, a taxa de crescimento folicular entre D8 e D10 foi maior para os grupos eCG e hCG 300. No entanto, mais vacas dos Grupos hCG 300 e hCG 200 SC apresentaram ovulação precoce. O tratamento com diferentes doses de hCG no D8 do protocolo de IATF não produziu efeitos semelhantes em relação à eCG. O terceiro estudo avaliou o efeito de eCG, FSH ou hCG na taxa de crescimento folicular, tamanho do folículo ovulatório, volume de CL e concentrações circulantes de E2 e P4, bem como o número de células lúteas grandes e pequenas em vacas submetidas a sincronização da ovulação. Foram utilizadas 17 vacas Nelore não lactantes em delineamento com dois quadrados latinos, totalizando oito réplicas. Os tratamentos com as gonadotrofinas eCG, FSH, ou hCG foram efetivos em aumentar a taxa de crescimento folicular entre D7-10 e consequentemente o diâmetro folicular no D10 e o diâmetro do folículo ovulatório em relação ao Controle. Além disso, o tratamento com diferentes gonadotrofinas aumentou o número de células lúteas grandes e pequenas sem, entretanto, se detectar diferenças no pico pré-ovulatório de E2, volume luteal e concentração circulante de P4 após a ovulação.

Palavras-chave: Bovino; Hormônio; Folículo; Corpo lúteo; Fertilidade

ABSTRACT

Ovarian function, steroid hormones and fertility in cows stimulated with gonadotropins

To increase profitability in beef and dairy cattle operations, adequate reproductive management strategies that provide high service and conception rates, especially at the beginning of the breeding season for beef cattle, and after the voluntary waiting period for dairy herds, are necessary. To achieve these goals various hormonal protocols have been developed to synchronize the emergence of a new follicular wave, estrus and ovulation, thus allowing fixed time artificial insemination (FTAI). Treatment with eCG has been included in FTAI protocols. Considering that eCG is an indispensable tool for reproductive management, a better understanding of its biological action in the final follicular growth process, ovulation and luteal development is crucial to optimize its use in hormonal protocols. At the same time, alternatives for eCG need to be tested. In this regard, it is important a better understanding of how FSH and LH act in the dominant follicle. Based on that, three studies were performed. The first study evaluated effects of eCG on fertility of 679 crossbred lactating grazing cows synchronized for FTAI. Treatment with eCG tended to increase P/AI at 30 and 60 days and increased P/AI at 30 and 60 days for cows inseminated at ≤ 70 DIM but had no effect in cows receiving AI after 70 DIM. The second study evaluated the effect of eCG or different doses of hCG on the final growth of the dominant follicle in 84 Nelore cows submitted FTAI. No differences were observed for the diameter of the largest follicle on D8 or D10. However, the growth rate of the dominant follicle between D8 and D10 was greater for the groups eCG and hCG 300. In addition, more cows from the Groups hCG 300 and hCG 200 SC presented premature ovulation. Treatment with different hCG doses on D8 of a FTAI protocol failed to produce similar effects compared to eCG. The third study evaluated the effect of eCG, FSH, or hCG on follicular growth rate, ovulatory follicle size, CL volume and circulating E2 and P4 concentrations, as well as the number of large and small luteal cells in cows submitted to a protocol for synchronization of ovulation. Seventeen non-lactating Nelore cows were used. Two Latin squares were done, totaling eight replicates. The gonadotropin treatments, eCG, FSH, or hCG, were effective in increasing the follicular growth rate between D7-10 and consequently the follicular diameter on D10 and ovulatory follicle diameter in comparison to Control. In addition, treatment with different gonadotropins increased the number of large and small luteal cells, however, there was no difference in preovulatory E2 peak concentration, CL volume and circulating P4 concentration post ovulation.

Keywords: Bovine; Hormone; Follicle; Corpus luteum; Fertility

1. INTRODUCTION

To increase profitability in beef and dairy cattle operations, adequate reproductive management strategies that provide high service and conception rates, especially at the beginning of the breeding season for beef cattle, and after the voluntary waiting period for dairy herds, are necessary. In order to achieve these goals various hormonal protocols have been developed to synchronize the emergence of a new follicular wave, estrus and ovulation, thus allowing fixed time artificial insemination (FTAI). These hormone treatments are based on GnRH and PGF2α (Pursley et al., 1995) or a combination of intravaginal progesterone devices (P4) and estradiol (E2; Bo et al., 1995). All of these protocols have been developed aiming to increase the speed with which cows are artificially inseminated postpartum, facilitating handling and increasing the reproductive efficiency.

In cattle, the beginning of the follicular wave, which coincides with a peak in blood concentrations of FSH, occurs when there is emergence of a group of antral follicles of similar size (4.0 mm diameter) (Ginther et al., 1996). According to Adams et al. (1993) the decline in responsiveness to FSH is associated with the moment that occurs follicle deviation, and this event is essential to the mechanism of selection of the dominant follicle.

The increase in blood concentrations of FSH observed 1-2 days before each follicular wave is considered critical to the beginning of the follicular waves during the estrous cycle (Adams et al., 1993; Ginther et al., 1998). When the largest follicle of the new follicular wave reaches 4-5 mm in diameter, FSH concentrations begin to decline and nearly 3 days later follicular deviation occurs, when the dominant follicle begins to depend on LH for its growth (Webb et al., 1999).

There is evidence in the literature that the acquisition of LH receptors (LHr) is the first step to maintain continuous growth of the selected follicle after the decrease in blood concentrations of FSH. The theca cells express LHr in preantral and preovulatory follicles (Xu et al., 1995; Beg et al., 2001; Luo et al., 2011). However, the LHr expression seems to occur only in granulosa cells from the follicles after the establishment of follicular dominance or deviation (Bodensteiner et al., 1995). Furthermore, Sartori et al. (2001) have shown that follicles from 7.0 to 8.5 mm in lactating Holstein cows had no ovulatory capacity in response to high doses of LH (40 mg, im) and ovulation was effective only in follicles ≥ 10 mm diameter. The authors suggest that although an increased LHr expression in granulosa cells is necessary for the acquisition of follicular dominance, only this increase is not sufficient to induce ovulatory capacity.

Treatment with gonadotropins such as equine chorionic gonadotropin (eCG) a glycoprotein with long half-life, produced by endometrial cups of pregnant mares between 40 and 130 days of gestation has been included in FTAI protocols with the aim to increase follicular development, ovulation and conception rate (Baruselli et al., 2012). Many studies report that eCG has similar activity as both FSH and LH, suggesting the ability to bind to receptors on the granulosa and theca cells, thus stimulating follicular growth (Murphy and Martinuk, 1991; Soumano et al., 1998; Yavas and Walton, 2000).

Studies with beef cows used eCG at a dose of 400 IU at the time of P4 removal aiming to stimulate the final follicular growth and increase the size and steroidogenic capacity of the resulting CL (Baruselli et al., 2004; Sá Filho et al., 2004). These authors observed that cows treated with eCG had higher blood P4 concentrations in the subsequent diestrus. Other studies with heifers and beef cows reported increased conception rates, especially in animals in anestrus or with low body condition who received eCG (Baruselli et al., 2003; Baruselli et al., 2004; Sá Filho et al., 2004 Hairstyle et al., 2005). Recent studies using high producing dairy cows observed increased CL volume and P4 concentrations during diestrus after FTAI in cows receiving eCG (400 IU). However, only cows with low body condition score (BCS) of those who received eCG had higher conception rate (Souza et al., 2009).

Despite the knownledge on the positive effect of eCG at the end of follicular development, poor is known about its biological action in bovine follicles. Considering that eCG is an indispensable tool for reproductive management, a better understanding of its biological action in the final follicular growth process, ovulation and luteal development is crucial to optimize its use in hormonal protocols. At the same time, alternatives for eCG need to be tested. That way it is important a better understanding of how FSH and LH act in the dominant follicle.

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2. EQUINE CHORIONIC GONADOTROPIN INCREASES FERTILITY OF GRAZING DAIRY COWS THAT RECEIVE FIXED-TIME ARTIFICIAL INSEMINATION IN THE EARLY BUT NOT LATER POSTPARTUM PERIOD

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ABSTRACT

This study evaluated effects of equine chorionic gonadotropin (eCG) on fertility of 679 crossbred (*Bos taurus* x *Bos indicus*) lactating grazing cows synchronized for fixed-time AI (FTAI). At a random day of the estrous cycle cows received an intravaginal progesterone (P4) implant, 2 mg estradiol benzoate (EB) and 100 µg gonadorelin (D0-AM). On D7-AM, cows received 150 µg d-cloprostenol and were randomly assigned into two treatments: eCG (n = 340; 400 IU eCG on D7), or Control (n = 339; no eCG). On D8-PM, P4 implants were removed and cows received 150 µg d-cloprostenol and 1 mg EB. Insemination was performed on D10-AM. Pregnancy was diagnosed 30 and 60 d after AI. Treatment with eCG tended to increase pregnancy per AI (P/AI) compared to Control at 30 (37.8 vs. 30.2%; P = 0.06) and 60 (31.9 vs. 25.1%; P = 0.08) d. Pregnancy loss and twinning did not differ between groups. Treatment with eCG increased (P < 0.05) P/AI at 30 (39.0 vs. 25.2%) and 60 (32.8 vs. 21.3%) d for cows inseminated at ≤ 70 d in milk (DIM) but had no effect in cows receiving AI after 70 DIM. Thus, eCG on D7 of a FTAI protocol increased fertility of crossbred dairy cows inseminated in the early postpartum period.

Keywords: Artificial insemination; Fertility; Hormone; Conception

2.1. Introduction

Reproductive efficiency is of economic importance for dairy herds worldwide because the lactation cycle is initiated and renewed by calving [1]. For most dairy herds it is crucial that cows become pregnant soon after the voluntary waiting period and this is particularly critical for herds with cattle that have lower persistency of milk production as lactation proceeds. Efficient use of artificial insemination (AI) can be an important tool to improve the genetics and future productivity of a herd as well as improve current reproduction and production of a

dairy herd. However, the efficacy of an AI program depends on timely and consistent submission of cows for AI which can be limited by factors such as low efficiency of heat detection and anovular cows [2,3]. In order to minimize these problems, protocols have been developed that synchronize ovarian function, hormonal dynamics, and produce a synchronized ovulation allowing fixed-time AI (FTAI) [3]. Use of FTAI in dairy herds can lead to more pregnant cows at a shorter interval after the end of the voluntary waiting period due to a reduced interval from calving to first service, thereby reducing the calving-conception period [4-7].

Ovulation synchronization programs generally utilize combinations of GnRH and PGF2α [8] or a combination of progesterone (P4) and estradiol (E2) [9]. Nevertheless, the percentage of cows that become pregnant to a single FTAI (P/AI) has frequently been sub-optimal in lactating dairy cows [10-12]. Thus, research that increases fertility during FTAI protocols could provide tools for dairy producers to improve reproductive efficiency of their herds.

In beef cattle, especially in *Bos indicus*, equine chorionic gonadotropin (eCG), a glycoprotein secreted by the endometrial cups of pregnant mares [13], has been used in P4/E2-based FTAI programs [14,15]. Treatment with eCG at the time of P4 implant removal improved preovulatory follicle growth, increased the percentage of cows ovulating at the end of a FTAI protocol, and increased P/AI, especially in cows or heifers with low body condition score (BCS) or in non-cycling cows [16-19]. The benefits of eCG on reproductive function are attributed to the long half-life of eCG and the dual FSH- and LH-like activity of the molecule [13].

In dairy cattle, few studies have evaluated the use of eCG during FTAI protocols and results have not shown the consistent advantages that have been reported for beef cattle. For example, Souza et al. [11] treated high producing dairy cows with eCG (400 IU) and reported an increase in CL volume and circulating P4 concentrations but no overall increase in P/AI due to eCG treatment. However, dairy cows with low BCS had an increase in P/AI in response to eCG in that study. Other studies reported no effect of eCG during a FTAI protocol on follicular and luteal dynamics or on P/AI in high producing Holstein cows [1,12]. Furthermore, there was no interaction between P/AI and other variables such as BCS and parity [1,12]. Nevertheless, two studies from New Zealand using grazing Friesian and Jersey-Friesian-cross dairy cattle reported positive effects of eCG on reproductive performance in anovular-anestrous cows that were synchronized for FTAI with P4/E2 [20] or GnRH-P4-

based [21] programs. In one of these studies, the positive effect of eCG was only observed in cows that were early postpartum (< 43 DIM) and not in cows that were later postpartum [21]. Thus, treatment with eCG during the final stages of FTAI protocols has been found to increase fertility in certain physiological situations, particularly in cows with lower BCS and earlier postpartum.

Crossbred cows (*Bos taurus* x *Bos indicus*) have been increasingly used for milk production in grazing systems in tropical countries to reduce the negative effects of heat stress and parasites on purebred Bos taurus dairy cattle. In this type of management system, the use of eCG during FTAI protocols may improve P/AI and thereby improve reproductive efficiency in these herds. Therefore, the objective of the present study was to evaluate the effect of eCG treatment on day 7 of the protocol on P/AI. Our hypothesis was that administration of 400 IU of eCG during the final stage of growth of the preovulatory follicle in a P4/E2-based FTAI protocol would increase P/AI of crossbred grazing dairy cows.

2.2. Material and methods

Cow management

This experiment was conducted in two commercial dairy farms located in Pitangui, MG, Southeast Brazil between October 2013 and July 2014. The climate of Pitangui is considered tropical with an average temperature of 21.8°C and annual rainfall of 1337 mm. The Animal Research Ethics Committee of Escola Superior de Agricultura “Luiz de Queiroz” (ESALQ)/University of São Paulo approved all procedures involving cows in this study.

A total of 679 crossbred Holstein x Gyr dairy cows was used, 492 multiparous and 187 primiparous, with an average milk production of 21.4 ± 7.6 kg/d, average days in milk (DIM) of 124.6 ± 97.0 (ranging from 38 – 498), lactation number of 2.46 ± 1.1 (ranging from 1 – 9), and number of AI of 2.1 ± 0.12 (ranging from 1 - 10). Different blood levels of crossbred were utilized and range from 1/2 to 31/32, being the first number for the Holstein bred and the percentage of each one are: 1/2 = 9.0%; 3/4 = 22.0%; 5/8 = 2.6%; 7/8 = 33.0%; 15/16 = 26.8% and 31/32 = 6.6%. Both farms used a grazing system with cows having continuous access to pastures of Monbaça guinea grass (*Panicum maximum* Jacq. ‘Monbaça’) with daily supplementation with a mixture of corn silage and a grain mix containing finely ground corn, soybean meal, citrus pulp, whole cottonseed, minerals, and vitamins. All cows had free access to water and were milked two times daily.

Experimental design

At a random day of the estrous cycle all cows received an intravaginal P4 implant (CIDR, Zoetis, São Paulo, Brazil), 2 mg estradiol benzoate (EB) i.m. (Gonadiol, MSD, São Paulo, Brazil), and 100 µg gonadorelin i.m. (Fertagyl, MSD, São Paulo, Brazil) in the morning (D0). Seven d later (D7) in the morning, all cows received 150 µg d-cloprostenol i.m. (Sincrocio, OuroFino, Cravinhos, Brazil) and were randomly assigned into two groups: eCG (n = 340) that received 400 IU eCG i.m. (Novormon, MSD, São Paulo, Brazil); and Control (n = 339) that did not receive eCG. On the afternoon of D8, P4 implants were removed and all cows received 150 µg d-cloprostenol i.m. and 1 mg EB i.m. The FTAI was performed at D10 in the morning (Fig. 1) by one of nine technicians with frozen-thawed proven semen. Pregnancy diagnoses were performed at 30 and 60 d after AI by transrectal ultrasound examination (DP-2200 VET, Mindray, Shenzhen, China). Calving data were collected accessing a software for dairy farm management (Ideagri, Belo Horizonte, Brazil), including twinning. Cows that died or were sold after the 60 d diagnoses were not used in future analyses.

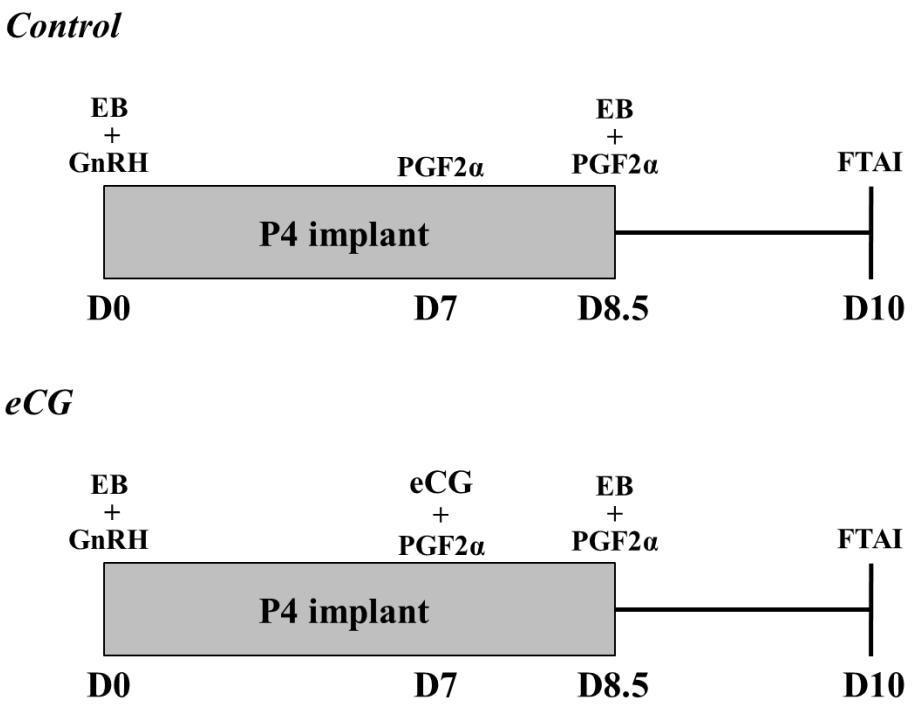

Figure 1. Experimental design to evaluate the effect of 400 IU eCG on Day 7 of the protocol on pregnancy per AI of crossbred dairy cows submitted to FTAI. D0 = 2 mg estradiol benzoate (EB), 100 µg gonadotropin-releasing hormone (GnRH) and progesterone implant

(P4); D7 = 0.5 mg prostaglandin F2α (PGF2α) and 400 IU equine chorionic gonadotropin (eCG); D8.5 = 1 mg EB and 0.5 mg PGF2α; D10 = fixed-time artificial insemination (FTAI).

Statistical analysis

Categorical data were analyzed by logistic regression using the GLIMMIX procedure of SAS version 9.4 (SAS/STAT, SAS Institute Inc., Cary, USA) fitting a binary distribution. The models included the fixed effects of treatment, parity, sire, technician, number of AI (first AI vs. resynchronized AI), categorized milk yield within parity in the month of AI as above or below the mean value, categorized DIM as above or below 70 d, the interactions between treatment and parity, treatment and number of AI, and the random effect of cow. The Kenward-Roger method was used to calculate the denominator degrees of freedom to approximate the F tests in the mixed models. Model fitting was evaluated using the fit statistics.

Milk yield, DIM, number of AI, and number of lactation were analyzed using the GLIMMIX procedure of SAS with models fitting a Gaussian distribution. Data were tested for normality of residuals. The model included the fixed effects of treatment, parity, and interaction between treatment and parity, and the random effects of cows. The Kenward-Roger method was used to calculate the denominator degrees of freedom to approximate the F tests in the mixed models.

In all analyses, only variables with $P < 0.20$ were kept in the final model, unless the variable was essential. Differences were considered significant when $P \leq 0.05$, whereas tendencies were considered when $0.10 \geq P > 0.05$.

2.3. Results

The results are presented as least squares means ± SEM. No differences between Control and eCG were observed for DIM (111.1 ± 5.8 vs. 120.7 ± 6.1; P = 0.25), number of lactations (2.4 ± 0.06 vs. 2.4 ± 0.06; P = 0.39), or previous AIs (2.1 ± 0.12 vs. 2.1 ± 0.12; P = 0.39).

The eCG group tended to increase P/AI at 30 (P = 0.06) and 60 (P = 0.08) d post AI. However, pregnancy loss between 30 and 60 d (P = 0.30) and between 60 d and calving (abortion rate) did not differ between groups (P = 0.56). In addition, no differences were

observed in the twinning rate between groups (P = 0.53; Table 1). Furthermore, there was a tendency (P = 0.10) for an interaction between the use of eCG and DIM. Cows treated with eCG and inseminated up to 70 d postpartum had greater P/AI at 30 (P = 0.02) and 60 (P = 0.05) d. However, in cows with more than 70 DIM there was no difference (P ≥ 0.74) between treatments (Fig. 2). In addition, no interaction was observed between the use of eCG and number of AI, ie, P/AI at 30 d for first AI postpartum [eCG = 38.6 ± 0.03 (69/184) vs. Control = 29.5 ± 0.03 (47/166); P = 0.41] and for the other services [eCG = 36.8 ± 0.04 (47/137) vs. Control = 34.0 ± 0.04 (50/159); P = 0.41] were different between treatments.

Table 1. Least squares means ± SEM of pregnancy per AI (P/AI), pregnancy loss, abortion rate, and twinning rate of crossbred dairy cows submitted to fixed-time artificial insemination with or without administration of 400 IU eCG on Day 7 of the protocol.

	eCG	Control	P-value
P/AI at 30 d, % (n/n)	37.8 ± 2.9 (133/340)	30.2 ± 2.9 (110/339)	0.06
P/AI at 60 d, % (n/n)	31.9 ± 2.8 (103/340)	25.1 ± 2.8 (88/339)	0.08
Pregnancy loss[1], % (n/n)	9.4 ± 2.9 (11/107)	14.4 ± 4.1 (13/94)	0.30
Abortion rate[2], % (n/n)	9.2 ± 4.1 (10/87)	12.2 ± 3.3 (10/78)	0.56
Twinning rate, % (n/n)	4.1 ± 2.5 (4/82)	2.4 ± 1.9 (2/72)	0.53

[1]Pregnancy loss between 30 and 60 d of gestation.
[2]Pregnancy loss between 60 d of gestation and calving.

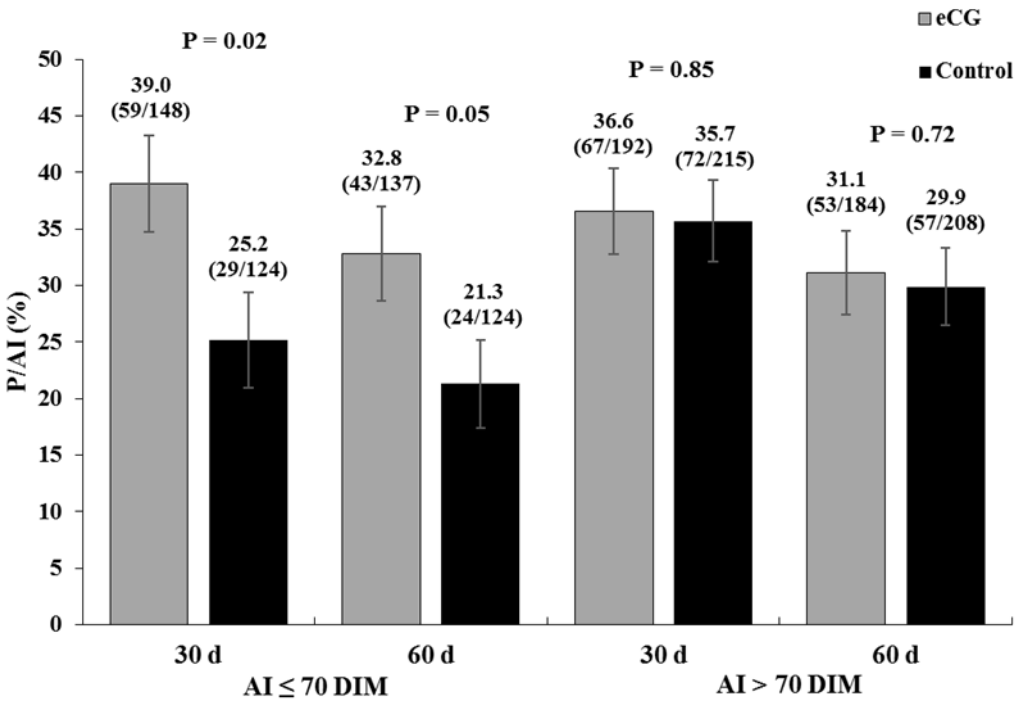

Figure 2. Interaction between days in milk (DIM) and equine chorionic gonadotropin (eCG) on P/AI of crossbred dairy cows (0) submitted to fixed-time artificial insemination earlier (AI ≤ 70 DIM) or later postpartum (AI > 70 DIM).

2.4. Discussion

The hypothesis that the administration of eCG on D7 of a synchronization protocol would increase fertility of crossbred (Bos taurus x Bos indicus) dairy cows submitted to FTAI was partially confirmed. Overall, there was a tendency to increase fertility, as measured by P/AI at 30 and 60 d after AI, in cows that received eCG compared to Control cows. Furthermore, it was found that the effect of eCG was due to an improvement in P/AI in cows that were earlier postpartum, before 70 DIM, and the effect of eCG was not observed for cows that were later in lactation. Thus, this research demonstrated the efficacy of eCG in crossbred Bos taurus x Bos indicus grazing dairy cattle but also indicated the group of cows, ≤ 70 DIM, that are most effectively treated with eCG.

Several studies have demonstrated the positive effects of eCG on fertility of beef cows, particularly in Bos indicus beef cows submitted to P4/E2-based protocol for FTAI [16-19,22]. However, treatment with eCG in high-producing Holstein dairy cows was either found to be ineffective [1,12] or only effective in cows with low BCS [11]. Other studies with anestrous, grazing dairy cattle (selected by lack of demonstration of estrus prior to the mating

season) reported a positive effect of eCG on fertility [20,21]. This study evaluated the use of eCG in crossbred Bos indicus x Bos taurus dairy cows (Gyr x Holstein) during a FTAI protocol. Our results were similar to results reported in Bos indicus [17,18,22], Bos taurus, or crossbred [19] beef cows and in grazing Bos taurus dairy cattle [20,21] in which treatment with eCG, near the time of P4 removal, increased P/AI during a FTAI protocol.

In general, previous studies have found that eCG treatment increased pregnancy rates of suckled beef cows mainly in herds exhibiting low BCS and/or high incidence of anovular condition at the beginning of the synchronization protocol [16,18,19,22], [23,24]. This effect was associated with enhanced follicle development and increased follicle diameter at FTAI leading to increased ovulation rate [17,18,24,25]. Similarly, previous positive results in New Zealand grazing dairy cattle were only found in studies that utilized only cows that were not cycling at the start of the breeding season [20,21]. Anovulation in these types of cattle are likely due to inadequate LH pulse frequency leading to inadequate growth of the dominant follicle and subsequent lack of ovulation [2,26]. For example, Sales et al. [18] reported that more than 84% of suckled Bos indicus cows were anovular at the beginning of the breeding season (30 to 60 d postpartum) and 33% of these cows had follicles smaller than 8 mm. Pessoa et al. [19] reported similar results with crossbred (Bos taurus x Bos indicus) suckled cows, with almost 79% of the cows between 40 and 70 d postpartum being anovular. Although, the percentage of cows that were anovular was not evaluated in the present study, previous studies with crossbred dairy cows have shown a relatively late resumption of cyclicity (~ 70 d postpartum [27]). In all of these physiological situations, it seems likely that treatment with eCG could increase gonadotropin support of follicle development, thereby increasing the size of the dominant follicle and likelihood of ovulation and by these means improving fertility in grazing purebred or crossbred beef and dairy cattle.

In contrast, in confined Bos taurus dairy herds, the number of cows that are anovular and the type of anovulation could be quite different [2] and this difference may underlie the lack of observed eCG effect in these situations. For example, ~ 75% of high producing dairy cows resumed cyclicity by 65 d postpartum [28-30]. In addition, 80% of anovular cows had follicles larger than 15 mm [31] suggesting that inadequate follicle growth may not underlie anovulation in many confined, high-producing dairy cattle. Indeed, LH pulses appear to be elevated in lactating dairy cattle [32]. Thus, inadequate gonadotropin support may not underlie anovulation in these physiological conditions and this may explain the lack of effect of eCG treatment on follicular and CL dynamics, or P/AI in studies with high producing

Holstein cows [1,12,33]. Anovular dairy cows in a grazing situation [20,21] or with a lower BCS (< 2.75) [11], may represent animals with inadequate LH support in which eCG treatment may improve follicle growth and fertility. Similarly, in our study, cows that are early postpartum may have inadequate gonadotropin support and therefore can demonstrate an improvement in fertility in response to eCG treatment. After recovering from the negative energy balance of the early postpartum period, these cows may no longer require supplemental gonadotropin support and therefore positive effects of eCG were not observed in cows after 70 DIM.

One difference in the protocol used in our study compared to other studies is that we gave eCG on the day before (D7 of protocol) removal of the P4 implant, whereas most studies gave eCG at the time of P4 implant removal (D8 or 9 of the protocol). This was done to extend the period of eCG action on the growing dominant follicle. The timing of eCG treatment at the end of the protocol has not yet been compared and therefore it is unclear if this protocol difference could contribute to our positive results compared to some other studies in dairy cattle. One of potential concern with this earlier treatment with eCG is a possible increase in double ovulation that could increase twinning rate. However, Melo et al. [34] found that a new follicular wave emerges 2 to 2.5 d after the same combination of hormones that we used at the beginning of the protocol (GnRH + EB + P4). Thus, it seems likely that follicle deviation, which occurs at 3 to 4 d after wave emergence, will already have occurred by D7 of the protocol [35,36]. In this study, we observed no increase in twinning rate due to eCG treatment (P = 0.53). Furthermore, pregnant cows with twin pregnancies can have increased pregnancy loss, mostly in the first trimester of gestation [37,38]. In our study, however, we observed no differences in early or later pregnancy loss between Control and eCG-treated cows. Future studies will be needed to assure that there is no change in double ovulation rate with this modified protocol, but we observed no evidence of this effect, based on lack of change in twinning or pregnancy loss in this study (Table 1).

2.5. Conclusions

In conclusion, the use of eCG on D7 of a FTAI protocol tended to increase fertility of crossbred grazing dairy cows due to an improvement in P/AI for cows inseminated at ≤ 70 DIM. Additionally, the use of eCG on D7 of the protocol did not increase twinning rate.

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3. EFFECT OF DIFFERENT CHORIONIC GONADOTROPINS ON FINAL GROWTH OF THE DOMINANT FOLLICLE IN *BOS INDICUS* COWS

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ABSTRACT

The aim of this study was to evaluate the effect of eCG or hCG on the final growth of the dominant follicle in Nelore (*Bos indicus*) cows submitted to fixed-time AI (FTAI). Eighty-four lactating cows with body condition score (BCS) of 2.9 (range 1-5) were used. At a random day of the estrous cycle (D0) cows received 2 mg estradiol benzoate and a reused intravaginal progesterone device (1.9 g). At D8, when the device was removed, 0.5 mg cloprostenol and 1 mg estradiol cypionate was given i.m., and cows were randomly assigned to receive on D8 one of the following treatments: Control (no treatment; n = 17), eCG (300 IU i.m.; n = 17), hCG 300 (300 IU i.m.; n = 18), hCG 200 IM (200 IU i.m.; n = 16) and hCG 200 SC (200 IU s.c.; n = 16). On the same day and 2 days later, cows were subjected to ovarian ultrasonography to evaluate the diameter of the largest follicle and to calculate follicular growth rate (D8 to D10). No differences were observed for the diameter of the largest follicle on D8 (P = 0.3) or D10 (P = 0.4) among treatments. However, the growth rate of the dominant follicle between D8 and D10 was greater for the groups eCG and hCG 300 and there were no differences between the other treatments (Control = 1.1 mm/day; eCG = 1.8 mm/day; hCG 300 = 1.8 mm/day; hCG 200 IM = 1.3 mm/day; hCG 200 SC = 1.3 mm/day; P = 0.02). In addition, more cows from the Group hCG 300 presented premature ovulation (44.4%) than cows from Control (5.8%), eCG (0%), or hCG 200 IM (12.5%), but did not differ from Group hCG 200 SC (18.7%). Regardless of treatment, the size of the largest follicle on D8 was different between cows that presented premature ovulation vs. cows that did not ovulate prematurely (11.3 mm vs. 9.9 mm, respectively; P = 0.01). Treatment with different hCG doses on D8 of a FTAI protocol failed to produce similar effects compared to eCG in terms of final follicular growth support and greater ovulatory follicle size. In addition, the groups hCG 300 and hCG 200 SC induced premature ovulation in a greater portion of cows. Thus, a single administration of hCG on D8 does not appear to be a reliable alternative to eCG treatment in FTAI protocols.

Keywords: Gonadotropin; eCG; hCG; Follicle; Cattle

3.1 Introduction

The majority of the cattle in tropical and subtropical countries that utilize pasture-based systems for beef production are zebu (*Bos indicus*) origin. This is probably due to greater adaptation and advantages to tropical grazing systems compared to *Bos taurus*, including better resistance to parasites and to high temperatures and humidity. Despite these physiological advantages, *Bos indicus* cows have longer periods of postpartum anovular condition, leading to low expression and detection of estrus and consequently low pregnancy rate at the end of the breading season [1].

To minimize the negative effects of the anovular condition and to ensure better conception rates during the breeding season, protocols for synchronization of ovulation for fixed-time artificial insemination (FTAI) based on estradiol (E2) and progesterone (P4) have been increasingly used [2-6].

Some physiological circumstances such as limited feed intake, low quality of feed, low body condition score (BCS) and calf presence during the postpartum period, lead to insufficient pulsatile release of LH to support the final growth of the dominant follicle and ovulation [7], compromising the results of FTAI programs [2,8,9]. Therefore, equine chorionic gonadotropin (eCG), has been successfully used in FTAI protocols by improving ovulation and pregnancy rates [9,10].

The eCG, a glycoprotein secreted by the endometrial cups of pregnant mares [11], is normally used at the time of P4 implant removal improving preovulatory follicle growth, percentage of cattle ovulating, and consequently pregnancy per AI (P/AI), especially in cows or heifers with low BCS or in anovular cows [8,12-15]. The benefits of eCG on reproductive function are attributed to the long half-life of eCG and the dual FSH- and LH-like activity of the molecule [11].

Human chorionic gonadotropin (hCG) is another gonadotropin produced by the human trophoblast and excreted in large quantities in the urine of pregnant women [16]. In cattle, hCG binds to LH receptors on granulosa and theca cells of ovarian follicles with high affinity [17,18] and has been used to induce ovulation in FTAI protocols [19-21] or to form accessory CL after AI [22-25]. In addition, hCG is a large molecule (~38000 Da) and is highly glycosylated, leading to elevated concentrations for a prolonged period after injection, with substantial amounts of hCG detected for more than 30 hours after treatment [26]. Surprisingly, we did not find any study in the literature that has tested hCG as stimulator of

dominant follicle growth during FTAI protocols. Thus, due to the LH activity and long half-life of hCG in cattle, it is of great interest to evaluate treatment with this hormone at the time of P4 device removal as an alternative treatment for eCG. The aim of this study was to evaluate the effect of eCG or hCG on the final growth of dominant follicle in Nelore cows submitted to FTAI and also to identify the threshold dose and route of administration of hCG. The hypothesis was that hCG would produce similar effects to the positive results observed with the use of eCG, improving preovulatory follicle growth and ovulatory follicle size in postpartum *Bos indicus* beef cows.

3.2 Material and methods

This experiment was conducted at the Experimental Station Hildegard Georgina Von Pritzelwiltz, located in Londrina, PR, Brazil. The Animal Research Ethics Committee of Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ)/University of São Paulo approved all procedures involving cows in this study (CEUA – 2015/01).

Cow management

Eighty-four Nelore lactating cows were used, with an average BCS of 2.9 ± 0.3 (range 1-5) and between 60-100 days postpartum. All cows were kept on pasture (*Brachiaria brizantha*), supplemented with mineral salt and had *ad libitum* access to water.

Experimental design

At a random day of the estrous cycle (D0) all cows received a previously used for 8 days intravaginal P4 implant (1.9 g, CIDR, Zoetis, São Paulo, Brazil) and 2 mg estradiol benzoate (EB) i.m. (Sincrodiol, Ourofino, Cravinhos, Brazil). Eight days later (D8), all cows received 0.5 mg d-cloprostenol i.m. (Estron, Tecnopec, São Paulo, Brazil), 1 mg estradiol cypionate (EC) i.m. (ECP, Zoetis, São Paulo, Brazil), and P4 implants were removed. At that time cows were randomly assigned into one of the five treatments: Control (n = 17) that did not receive any other treatment; eCG (n = 17) that received 300 IU eCG i.m. (Novormon, MSD, São Paulo, Brazil) on D8; hCG 300 (n = 18) that received 300 IU hCG i.m. (Chorulon, MSD, São Paulo, Brazil) on D8; hCG 200 IM (n = 16) that received 200 IU hCG i.m. on D8; and hCG 200 SC (n = 16) that received 200 IU hCG s.c. on D8 (Figure 1).

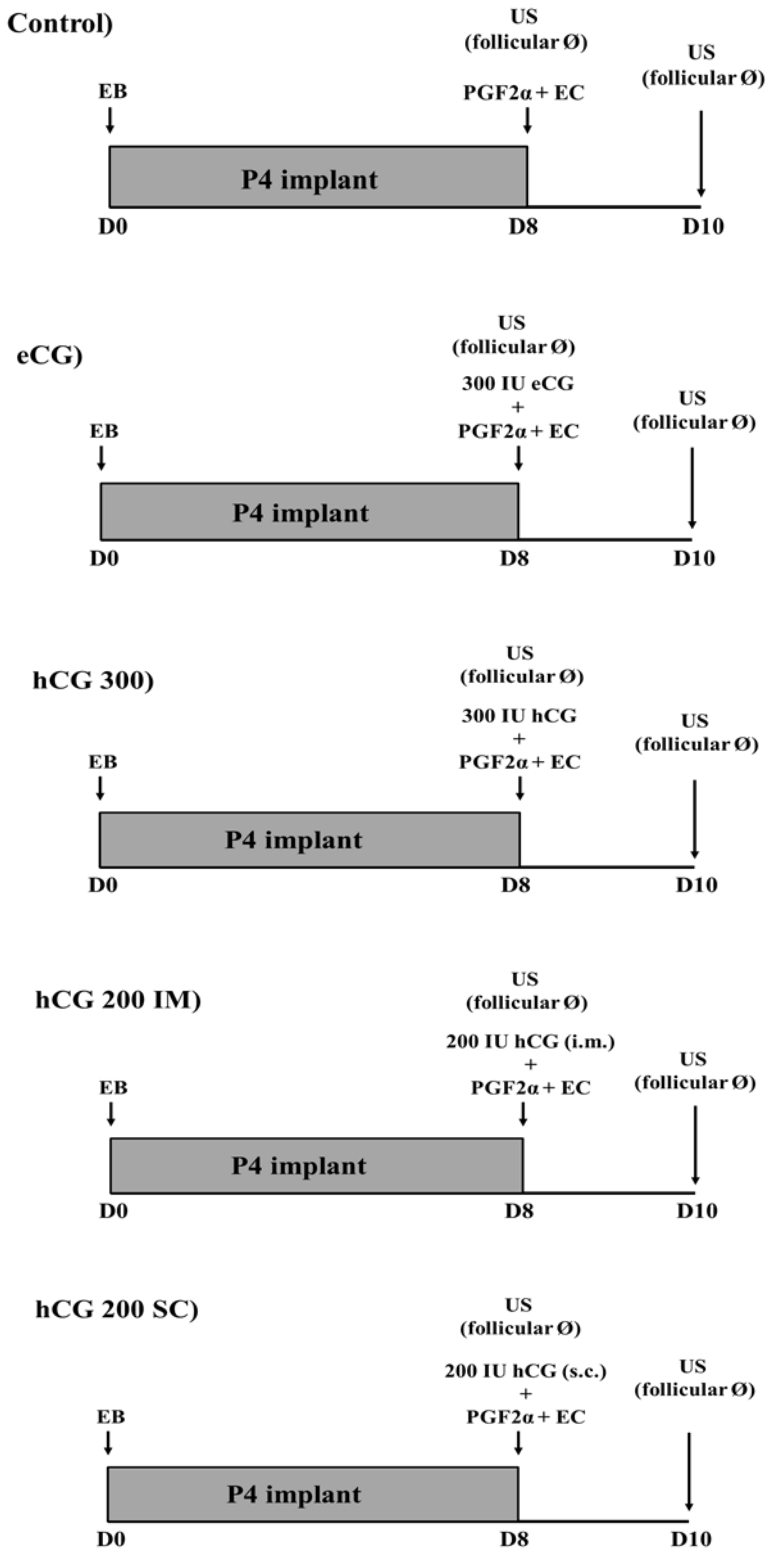

Figure 1. Experimental design to evaluate the effect of eCG or hCG on the final growth of dominant follicle in Nelore cows submitted to protocol of synchronization of ovulation. D0 = 2 mg estradiol benzoate (EB) and progesterone implant (P4); D8 = 0.5 mg prostaglandin F2α (PGF2α), 1 mg estradiol cypionate (EC) and 300 IU of equine chorionic gonadotropin (eCG)

or different doses of human chorionic gonadotropin (hCG). US = transrectal ultrasound; Ø = diameter.

Ultrasound examinations

All cows were submitted to transrectal ultrasound examination (DP-2200 VET, Mindray, Shenzhen, China) of the ovaries on days 8 and 10 (48 hours later) to evaluate the diameter of the largest follicle and to calculate follicular growth rate between D8 and D10. Also, cows that had at least one follicle greater than 8 mm on D8 and absence of an ovulatory follicle on D10 were considered having had premature ovulation.

Statistical analysis

Data not normally distributed were transformed to natural logarithms. The Statistical Analysis System (SAS) was used with a MIXED procedure and a REPEATED statement to minimize autocorrelation (Version 9.4; SAS Institute Inc., Cary, NC, USA). For difference between treatments on D8, D10, and follicle growth one-way analysis of variance was used. Differences in frequency of ovulation among groups were examined by chi-square. A probability of $P \leq 0.05$ indicated a difference was significant. Data are presented as least squares means ± SEM, unless otherwise indicated.

3.3 Results

No differences were observed for the diameter of the largest follicle at D8 ($P = 0.3$) or D10 ($P = 0.4$) among treatments (Table 1). However, the growth rate of the dominant follicle between D8 and D10 was higher for the groups eCG and hCG 300, and there were no difference between the other treatments ($P = 0.02$; Table 1). In addition, the group hCG 300 had more premature ovulations than groups Control, eCG and hCG 200 IM, but did not differ from group hCG 200 SC ($P = 0.01$; Figure 2). Regardless of treatment, the size of the largest follicle on D8 was different between cows with premature vs. normal ovulation (11.3 mm vs. 9.9 mm, respectively; $P = 0.01$).

Table 1. Least squares means ± SEM of the largest follicle (LF) diameter on days 8 and 10 and the growth rate between day 8 and 10 of Nelore cows submitted to protocol of synchronization of ovulation and stimulated with different gonadotropins.

	LF diameter on D8, mm	LF diameter on D10, mm	Growth rate of the LF, mm/day
Control (n = 17)	10.3 ± 0.6	12.7 ± 0.6	1.1 ± 0.1[b]
eCG (n =17)	9.9 ± 0.6	13.5 ± 0.6	1.8 ± 0.2[a]
hCG 300 (n = 18)	10.3 ± 0.5	12.8 ± 0.4	1.8 ± 0.1[a]
hCG 200 IM (n = 16)	9.4 ± 0.6	12.0 ± 0.7	1.3 ± 0.1[b]
hCG 250 SC (n = 16)	11.1 ± 0.4	13.5 ± 0.6	1.3 ± 0.1[b]
P-value	0.3	0.4	0.02

[a, b] Least squares means differed among treatments (P < 0.05)

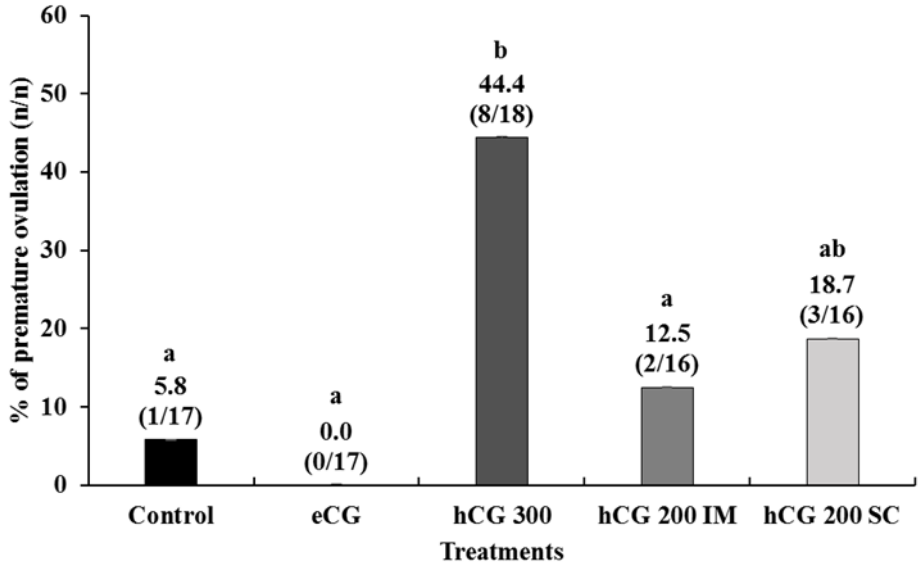

Figure 2. Percentage of premature ovulation of Nelore cows submitted to protocol for synchronization of ovulation and stimulated with different gonadotropins.

3.4 Discussion

This study demonstrated the effect of eCG or different doses or routes of administration of hCG on ovarian follicular growth of postpartum *Bos indicus* beef cows submitted to P4/E2-based protocols. The hypothesis that the administration of hCG on D8 of

a synchronization protocol would increase preovulatory follicle growth and produce bigger ovulatory follicles, similar to those induced by treatment with eCG was not confirmed.

Follicular growth rate from days 8 to 10 was higher for cows treated with eCG compared to Control. These findings were consistent with most of the studies in the literature in which suckling *Bos indicus* or *Bos taurus* beef cows treated with eCG presented higher growth rates [8,13-15]. The FSH- and LH-like activity of eCG has been demonstrated [7], thus the effect of eCG on follicular development, could explain the greater growth rate. Despite that, no difference was observed on the diameter of the LF on D10 and this is probably due to the insufficient number of cows in each group and for the variation of the LF size on day 8 (6-7,9 mm = 15; 8-9.9 mm = 15; 10-11.9 mm = 35; 12-15 mm = 19).

Therefore, our main finding was that treatment with 200 hCG IM or 200 hCG SC failed to enhance follicular growth rate and follicle size on D10. As shown in Table 1, both treatments presented the same follicular growth as the Control group. It seems that only one treatment with these dosages of hCG is not enough to increase follicle growth, probably due to the shorter period of action compared to eCG. Furthermore, despite no differences in premature ovulation rate, more data are necessary to prove that these hCG treatments do not cause early ovulation. In addittion, the group hCG 300 increased follicular growth rate, presenting the same growth rate as eCG (1.8 mm/day), however 44.4% of the cows on this group had premature ovulation. Not surprisingly, all cows that have had premature ovulation presented follicles greater than 10 mm on D8, that is, a dominant follicle with a greater ovulatory capacity in response to an exogenous stimulus [27]. Thus, although this dose of hCG stimulates a faster follicle growth, it is not low enough to prevent premature ovulation in dominant follicles. Therefore, multiple treatments with smaller doses of hCG once a day or using slower release vehicles might solve this problem.

In Nelore cows, follicular deviation happened when the largest follicle reached 7.0 to 7.5 mm of diameter [28] and one of the major characteristics of the dominant follicle is the presence of LH receptors in the granulosa cells [29-32]. Moreover, Gimenes et al. [27] reported that administration of 25 mg pLH induced ovulation in 33.3, 80.0, and 90.0% of Nelore heifers with follicles that were 7.0 to 8.4, 8.5 to 10, and > 10 mm in diameter, respectively. Based on that and on the diameter of the largest follicle on D8 for the cows that had premature ovulation (11.3 mm), and that more than 85% of these cows were on this condition, we can speculate hCG treatments, even in small doses, induced premature ovulation. In addition, Giordano et al. [26] showed that LH concentrations after hCG

treatment remained elevated for at least 30 hours and reached a peak, on average, 9 hours after treatment. Therefore, it is likely that hCG induced ovulation instead of stimulated faster follicle growth. At estrus, E2 reaches the peak and leads to the LH peak. Immediately after this LH peak, circulating concentration of E2 rapidly decreases [33]. Furthermore, one characteristic of early stages of ovulation and luteinization of the dominant follicle is reduction of E2 concentrations [34-36]. Thus, hCG treatment is probably leading to an ovarian dysfunction, by promoting a premature LH-like peak, with subsequent decrease in circulating E2, not allowing for an ideal final follicle development.

3.5 Conclusions

In conclusion, different hCG treatments or routes of administration on D8 of a FTAI protocol failed to produce similar effects compared to eCG in terms of final follicular growth support and greater ovulatory follicle size. In addition, the groups hCG 300 and hCG 200 SC induced premature ovulation in a substantial number of cows. Thus, a single administration of hCG on D8 does not appear to be a reliable alternative to eCG treatment in FTAI protocols.

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4. EFFECT OF GONADOTROPINS ON DEVELOPMENT OF THE OVULATORY FOLLICLE AND RESULTING CORPUS LUTEUM IN COWS

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ABSTRACT

The aim of this study was to evaluate the effect of eCG, FSH, or hCG on follicular growth rate, ovulatory follicle size, CL volume and circulating E2 and P4 concentrations, as well as the number of large and small luteal cells in cows submitted to a protocol for synchronization of ovulation. Seventeen non-lactating Nelore cows with body condition score (BCS) of 2.7 ± 0.4 (scale of 1 to 5) were used. At the beginning of the protocol (D0) all cows received 2 mg i.m. estradiol benzoate, 25 mg i.m. PGF2α, and an intravaginal P4 device. Cows received 25 mg i.m. PGF2α on D7 and had P4 implant removal plus 25 mg i.m. PGF2α on D8. On D7, cows were randomly assigned into four groups: Control = No gonadotropin treatment; eCG = 300 IU i.m. eCG on D7; FSH = 20 mg FSH on D7, D8, and D9 (divided into two daily treatments of 10 mg i.m., 12 hours apart); hCG = 200 IU i.m. hCG on D7 and 100 IU i.m. hCG on D8 and D9. Ultrasound examinations and blood sampling were performed daily until the 7th day post ovulation, when CL biopsies were performed. Two Latin squares were done, totaling eight replicates. The results are presented as least squares means ± SE following the group order Control, eCG, FSH, and hCG. As expected, there was no difference in follicle diameter (mm) on D7 (7.7 ± 0.3, 7.9 ± 0.3, 7.7 ± 0.3, and 7.5 ± 0.3; $P > 0.05$). However, on D10 follicle diameters of groups eCG, FSH and hCG were larger than Control (11.3 ± 0.3, 12.5 ± 0.3, 12.5 ± 0.3, and 12.6 ± 0.3; $P < 0.05$) with greater follicular growth rates (mm/day) between D7-10 (1.2 ± 0.3; 1.5 ± 0.3, 1.6 ± 0.3, and 1.7 ± 0.3; $P < 0.03$) in relation to Control. Similarly, the diameter of the ovulatory follicle (12.9 ± 0.3, 13.6 ± 0.3,

13.6 ± 0.3, and 13.8 ± 0.3; P < 0.05) was smaller for Control, but the E2 peak concentration was not different (8.7 ± 0.4, 10.2 ± 0.6, 9.8 ± 0.6, and 8.5 ± 0.4 pg/mL; P > 0.05). Seven days post ovulation, CL volume (3.88 ± 0.26, 4.53 ± 0.26, 3.80 ± 0.31, and 4.44 ± 0.28 cm^3; P > 0.05) and circulating P4 concentration (2.9 ± 0.3, 3.6 ± 0.3, 2.9 ± 0.3, and 3.5 ± 0.3 ng/mL; P > 0.05) were not different among groups. Nevertheless, the groups treated with gonadotropins had a greater number of large (224 x 10^7 ± 610, 468 x 10^7 ± 558, 586 x 10^7 ± 588, and 519 x 10^7 ± 541; P < 0.01) and small (238 x 10^8 ± 688, 478 x 10^8 ± 629, 484 x 10^8 ± 717, and 555 x 10^8 ± 579; P < 0.01) luteal cells than the Control group. In conclusion, each of the gonadotropin treatments, eCG, FSH, or hCG, were effective in increasing the follicular growth rate between D7-10 and consequently the follicular diameter on D10 and ovulatory follicle diameter. In addition, treatment with different gonadotropins increased the number of large and small luteal cells, however, there was no difference in E2 peak concentration, CL volume and circulating P4 concentration post ovulation.

Keywords: eCG; FSH; hCG; Bovine; Follicle; CL

4.1 Introduction

It is well known that gonadotropins such as FSH and LH are essential for follicle development in cattle. According to Webb et al. [1] and Bergfelt et al. [2], when the biggest follicle of a new follicular wave reaches the size of 5 mm of diameter, circulating FSH concentration starts to decline, and 3 days later, deviation occurs. At this moment, the dominant follicle depends mainly of LH to grow. However, at least some circulating FSH after deviation is still necessary for continued growth of the dominant follicle [2].

Most of beef cows in grazing systems present anovular condition after parturition due to inadequate concentrations of gonadotropins and metabolic hormones caused by insufficient feed intake and the suckling stimulus of the calf (reviewed by Wiltbank et al. [3]). To minimize this situation, protocols that synchronize ovarian function, hormonal dynamics, and produce a synchronized ovulation allowing fixed-time AI (FTAI) have been used [4,5]. To improve follicle growth, ovulation and pregnancy rates, equine chorionic gonadotropin (eCG) has been used, especially in progesterone (P4)/estradiol (E2)-based protocols [6-8]. The benefits of eCG on reproductive functions are attributed to the dual FSH- and LH-like activity and the long half-life of the molecule [9]. Normally, eCG is given at the time of P4 device removal, leading to an extra gonadotropin support for the preovulatory follicle [4,8,10]. Furthermore, some studies reported that cows and heifers treated with eCG presented greater circulating P4 concentrations during the subsequent luteal phase [4,11], probably due to bigger CL volume and modified specific steroidogenesis-based features (mitochondrial shape and number of large luteal cell) [12].

Alternatives for the use of eCG are being tested, however without success. One of the alternatives most tested is the porcine FSH (pFSH). According to Fortune et al. [13], granulosa cells of the dominant follicle present a great number of FSH receptors and supplementation with this hormone could stimulate follicle growth. Despite that, studies using supplementation with pFSH at the time of P4 implant removal provided inconsistent results. For example, supplementation with 10 mg of FSH failed to increase final follicle growth, ovulation rate or pregnancy per AI (P/AI) in comparison with treatment with eCG or calf removal [6,14]. In two other studies, supplementation with 20 mg of FSH did not improve P/AI in anovular *Bos indicus* [15] and *Bos taurus* [16] beef cows compared to Control. These results are probably due to the short time period (16 hours) that FSH treatment can sustain active physiological concentrations in cattle [17,18].

Another gonadotropin that has been tested is the human chorionic gonadotropin (hCG). In cattle, hCG binds to LH receptors on granulosa and theca cells of ovarian follicles with high affinity [19,20] and substantial amounts of hCG can be detected for more than 30 hours after treatment [21]. It has been used to induce ovulation in FTAI protocols [22-24] or to create accessory CL after AI [25-28]. In the best of our knowledge, only one study in the literature has tested hCG as a stimulator of dominant follicle growth during FTAI protocols (Prata et al., 2018; submmited to Theriogenology). According to these authors, only one treatment with 200 IU i.m. or 200 IU s.c. hCG did not stimulate a faster growth of the dominant follicle as a treatment with 300 IU i.m. eCG did. Moreover, treatment with 300 IU i.m. hCG induced premature ovulation in a relatively high percentage (44.4%) of cows.

Based on the data presented above, we can conclude that only one treatment with FSH or hCG does not seem to be enough to match the stimulation provided by eCG on the development of the preovulatory follicle. Thus, the objective of this study was to evaluate the effect of eCG or multiple treatments of FSH or hCG on ovarian dynamics and reproductive hormones pre- and post-ovulation, as well as the number of large and small luteal cells in Nelore cows submitted to protocols for synchronization of ovulation. We hypothesized that treatments with eCG, FSH or hCG would produce greater follicle growth rates and ovulatory follicle sizes, consequently greater peaks of E2, larger CL post ovulation, greater P4 concentrations and more large luteal cells (LLC), than the not treated Control group.

4.2 Material and methods

This experiment was conducted at the University of São Paulo, College of Agriculture 'Luiz de Queiroz' (ESALQ, Piracicaba, São Paulo, Brazil). The Animal Research Ethics Committee of ESALQ approved all procedures involving cows in this study (Protocol number: CEUA – 2015/01).

Cow management

Seventeen non-lactating Nelore cows with BCS of 2.7 ± 0.4 (scale of 1 to 5) were used. Cows were housed in pens (4 to 5 per pen) and were feed a diet based on hay of Tifton and supplemented with a grain mix of finely ground corn, soybean meal, citrus pulp, minerals and vitamins to maintain body weight according to the National Research Council [29] and had free access to water.

Experimental design

At the beginning of the protocol (D0) all cows received 2 mg i.m. estradiol benzoate (Gonadiol, Zoetis, São Paulo, Brazil), 25 mg i.m. dinoprost tromethamine (PGF2α, Lutalyse, Zoetis, São Paulo, Brazil), and an intravaginal P4 device (CIDR, Zoetis, São Paulo, Brazil). Seven days later (D7) all cows received 25 mg i.m. PGF2α and on D8 another PGF2α treatment was performed and cows had CIDR removal. On D7, cows were randomly assigned into four groups: Control = No gonadotropin treatment; eCG = 300 IU i.m. eCG (Novormon, Zoetis, São Paulo, Brazil) on D7; FSH = 20 mg FSH on D7, D8 and D9 (divided into two daily treatments of 10 mg i.m., 12 hours apart; Folltropin-V, Bioniche, Belleville, Canada); hCG = 200 IU i.m. hCG (Chorulon, MSD, São Paulo, Brazil) on D7 and 100 IU i.m. of hCG on D8 and D9 (Figure 1). The study was performed in a Latin Square design and to increase the number of cows in each treatment. Two Latin Squares were performed, totaling eight replicates.

Ultrasound examinations

Daily transrectal ultrasound examinations (DP-2200 VET, Mindray, Shenzhen, China) of the ovaries for measurement of follicles and CL were performed throughout the study until the seventh day post-ovulation using a 7.5 MHz linear transducer. Maps of the ovaries were drawn for each individual cow, and size and position of follicles ≥ 4 mm in diameter and CL were recorded. Data from cows with follicles > 5 mm during the protocol were excluded of the analyses. Occurrence of ovulation was characterized by the absence of a previously dominant follicle and confirmed by the appearance of a CL 3 days later and multiple

ovulation was considered when more than one follicle ovulated. Also, cows that had at least one follicle greater than 8 mm on D8 and absence of an ovulatory follicle on D9 were considered having had premature ovulation. Furthermore, for cows that did not ovulate, we utilized the measurements until the last day before follicle became atretic. Seven days after ovulation, CL volume was calculated with the formula $V = 4/3 \times \pi \times R^3$ using a radius (R) calculated by the formula R = (length/2 + width/2)/2. For a CL with a fluid-filled cavity, the volume of the cavity was calculated and subtracted from the total volume of the CL.

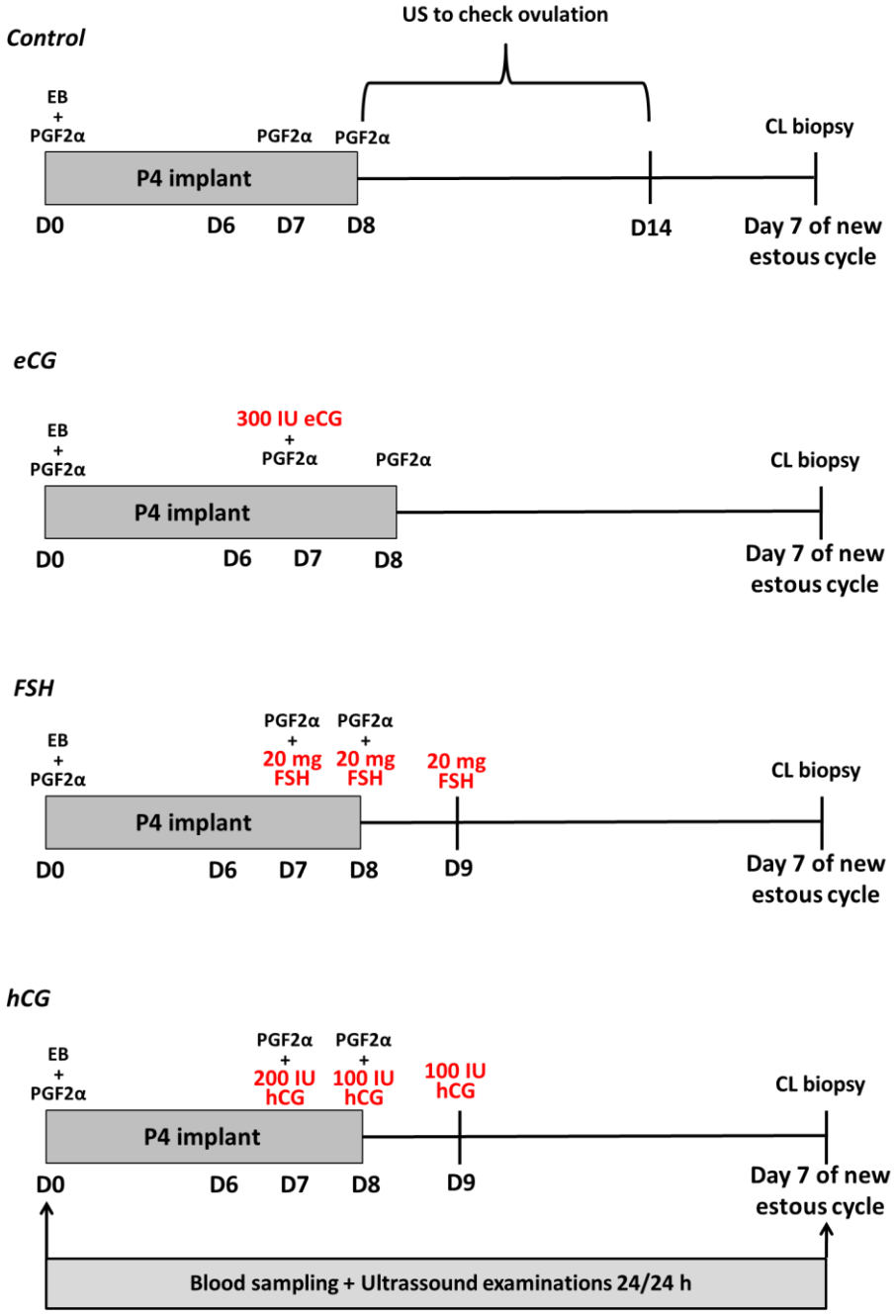

Figure 1. Experimental design to evaluate the effect of different gonadotropins on the final growth of dominant follicle, CL development and histology post ovulation and reproductive hormones in Nelore cows submitted to protocols of synchronization of ovulation. EB = estradiol benzoate; P4 implant = progesterone intravaginal device; PGF2α = prostaglandin F2α, eCG = equine chorionic gonadotropin; FSH = follicle stimulating hormone; hCG = human chorionic gonadotropin.

Blood samples and analyses of circulating progesterone and estradiol

Approximately, 9 mL of blood was collected daily by puncture of the jugular vein utilizing Vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, USA). After collection, samples were placed in ice and transported to the laboratory. Blood tubes were centrifuged at 1,900 x *g* for 15 min at 4^{o}C and serum was frozen at -20^{o}C for further analyses. Concentrations of P4 were analyzed by RIA using a commercial kit (ImmuChem, MP Diagnostics, USA). The intra- and inter-assay coefficient of variations were 5.6% and 11.1%, respectively. Concentrations of E2 were analyzed by a modified RIA [30] and the intra- and inter assay coefficient of variation were 6.1% and 2.3% respectively.

CL biopsies

Seven days after ovulation, CL biopsies were collected by means of transvaginal ultrasonography with a 7.5 MHz convex transducer (DP-2200 VET, Mindray, Shenzhen, China) coupled with as aspiration guide [31]. The CL fragment was immediately submerged in 4% paraformaldehyde for tissue fixation and conventional embedding procedures. Paraffin blocks were cut into 2 µm sections using a microtome (Leica RM 2125 RT, Germany), and sections were stained with hematoxylin-eosin for observation under a light microscope (Olympus BX 60, Olympus, Tokyo, Japan) at magnification x400. Large and small luteal cells were counted according to Rigoglio et al. [12].

Statistical analyses

The Statistical Analysis System version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC) was used to perform the statistical analyses.

The fixed effects of BCS, replicate, treatment and interactions were included in the model. Variables that did not present interaction ($P < 0.10$) were excluded from the statistical model. Cow within replicate was included in the model as aleatory effect.

Continuous variables were analyzed with models respecting a Gaussian distribution. Data not normally distributed were transformed to natural logarithm. All of them were submitted to MIXED procedure. However, for in time repeated measures as follicular dynamics and P4, the statement REPEATED were added.

Binomial data, such as ovulation rate, percentages of premature ovulation and multiple ovulation were analyzed by the GLIMMIX procedure, and differences in frequency among groups were examined by chi-square.

Comparisons were performed using means adjusted by the least squares method and are presented as least squares means ± SEM, with the distributions in the original scales. Values with $P \leq 0.05$ were considered significant.

4.3 Results

No differences were observed for the day of follicular wave emergence, and diameter of the largest follicle on D6 and D7 (Table 1). However, on D8 the eCG and FSH groups had larger follicles than the Control group, but did not differ from the hCG group. The follicular diameter on D9 and D10 and the maximal size of the ovulatory follicle (only cows that ovulated) were larger for gonadotropin-treated cows. However, when we analyzed the maximal size of the dominant follicle (all cows), cows from the hCG group had larger follicles than from the other groups (Table 1). Furthermore, the eCG group ovulated earlier than Control, but did not differ from FSH and hCG, and cows from the groups Control and hCG needed more time to reach the maximal diameter of the dominant follicle compared to eCG (Table 1). Although the gonadotropin treatments had a larger ovulatory follicle, no differences were observed for circulating E2 concentrations (Figure 2), CL volume (Table 1) and circulating P4 concentrations (Figure 3). Despite that, gonadotropin-treated cows had more small and large luteal cells than Control (Figure 4).

Regarding follicular growth rate, gonadotropin treatments induced greater growth rates between D7-8 and D7-10 compared to Control (Table 2). The growth rate between D9-10 was greater from hCG than Control, but did not differ from eCG or FSH. Moreover, no difference was observed for ovulation rate, but hCG treatment induced more premature ovulations than the other groups, and FSH-treated cows presented higher multiple ovulation rate (Table 3).

Table 1. Least squares means ± SEM of follicular wave emergence, diameter of the largest follicle on days 6, 7, 8, 9 and 10, diameter of ovulatory follicle and CL volume 7 days after ovulation of Nelore cows synchronized for ovulation and submitted to different gonadotropin treatments.

	Control (n = 30)	eCG (n = 30)	FSH (n = 31)	hCG (n =30)
Follicular wave emergence, day	3.5 ± 0.1	3.4 ± 0.1	3.6 ± 0.1	3.3 ± 0.1
Diameter of the largest follicle on D6, mm	6.9 ± 0.3	6.9 ± 0.3	6.6 ± 0.3	6.5 ± 0.3
Diameter of the largest follicle on D7, mm	7.7 ± 0.3	7.9 ± 0.3	7.7 ± 0.3	7.5 ± 0.3
Diameter of the largest follicle on D8, mm	8.7 ± 0.3^{a}	9.6 ± 0.3^{b}	9.4 ± 0.3^{b}	9.0 ± 0.3^{ab}
Diameter of the largest follicle on D9, mm	10.0 ± 0.3^{a}	11.0 ± 0.3^{b}	10.8 ± 0.3^{b}	10.5 ± 0.3^{b}
Diameter of the largest follicle on D10, mm	11.3 ± 0.3^{a}	12.5 ± 0.3^{b}	12.5 ± 0.3^{b}	12.6 ± 0.3^{b}
Maximum size of ovulatory follicle, mm	12.9 ± 0.3^{a}	13.6 ± 0.3^{b}	13.6 ± 0.3^{b}	13.8 ± 0.3^{b}
Day of ovulation, day	12.5 ± 1.8^{a}	11.6 ± 1.8^{b}	11.7 ± 1.9^{ab}	12.2 ± 2.0^{ab}
Maximum size of dominant follicle, mm	13.0 ± 0.3^{a}	13.3 ± 0.3^{a}	13.3 ± 0.3^{a}	14.4 ± 0.3^{b}
Day of maximum dominant follicle diameter, day	12.6 ± 1.9^{a}	11.6 ± 1.9^{b}	11.9 ± 2.0^{b}	12.5 ± 2.0^{a}
CL volume, cm^3	3.88 ± 0.26	4.53 ± 0.26	3.80 ± 0.31	4.44 ± 0.28

^{a,b,} $P < 0.05$

Table 2. Least squares means ± SEM of follicular growth rate between days 6 and 11of Nelore cows synchronized for ovulation and submitted to different gonadotropin treatments.

	Control (n = 30)	eCG (n = 30)	FSH (n = 31)	hCG (n = 30)
Follicle growth rate D6-7, mm/day	0.7 ± 0.4	1.0 ± 0.4	1.1 ± 0.4	1.0 ± 0.4
Follicle growth rate D7-8, mm/day	1.0 ± 0.1[a]	1.6 ± 0.1[b]	1.7 ± 0.1[b]	1.5 ± 0.1[b]
Follicle growth rate D8-9, mm/day	1.6 ± 0.3	1.7 ± 0.3	1.7 ± 0.3	1.9 ± 0.3
Follicle growth rate D9-10, mm/day	1.2 ± 0.1[a]	1.5 ± 0.1[ab]	1.7 ± 0.1[ab]	2.0 ± 0.1[b]
Follicle growth rate D10-11, mm/day	0.9 ± 0.1[ab]	0.9 ± 0.1[ab]	0.7 ± 0.1[b]	1.3 ± 0.1[a]
Follicle growth rate D7-10, mm/day	1.2 ± 0.3[a]	1.5 ± 0.3[b]	1.6 ± 0.3[b]	1.7 ± 0.3[b]

[a,b]$P < 0.05$

Table 3. Least squares means ± SEM of ovulation, premature ovulation and multiple ovulation rates of Nelore cows synchronized for ovulation and submitted to different gonadotropin treatments.

	Control (n = 30)	eCG (n = 30)	FSH (n = 31)	hCG (n = 30)
Ovulation rate, % (n/n)	83.3 ± 6.9 (25/30)	90.0 ± 5.5 (27/30)	80.6 ± 7.2 (25/31)	83.3 ± 6.9 (25/30)
Premature ovulation rate, % (n/n)	0.0[a] (0/25)	0.0[a] (0/27)	0.0[a] (0/25)	12.0 ± 6.6[b] (3/25)
Multiple ovulation rate, % (n/n)	0.0[a] (0/25)	3.7 ± 3.7[a] (1/27)	28.0 ± 9.1[b] (7/25)	0.0[a] (0/25)

[a,b]$P < 0.05$

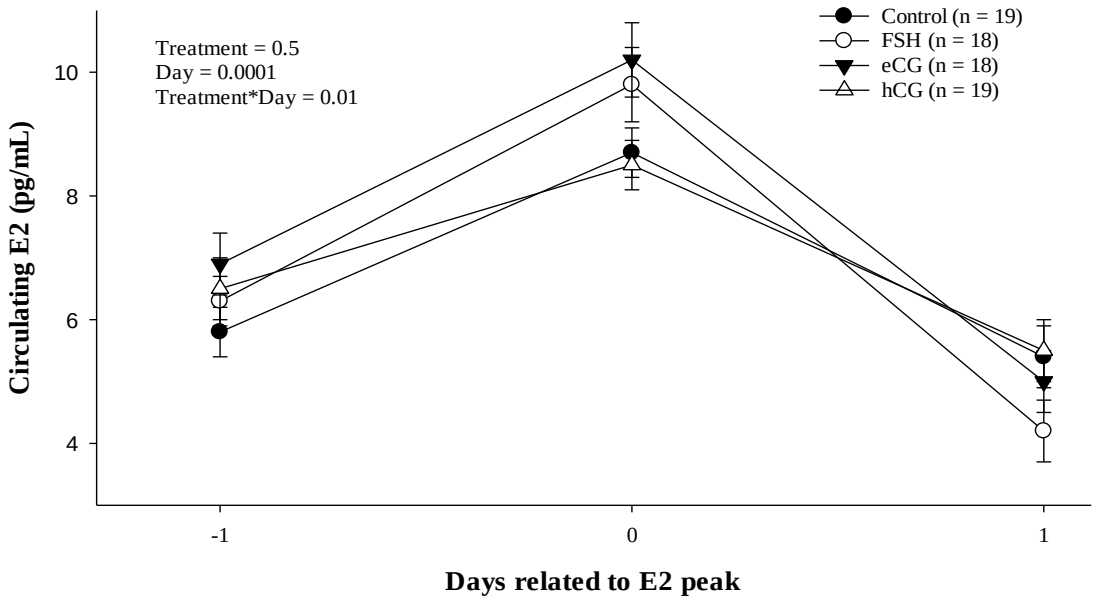

Figure 2. Least squares means ± SEM of circulating estradiol concentration normalized to the time of the peak in Nelore cows synchronized for ovulation and submitted to different gonadotropin treatments.

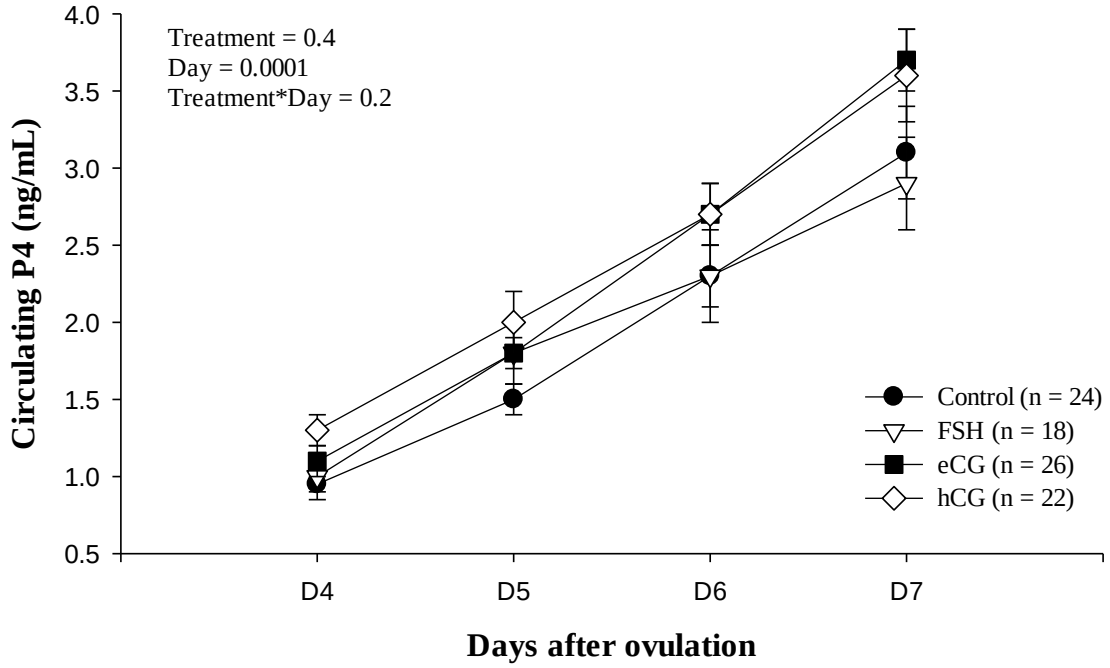

Figure 3. Least squares means ± SEM of circulating progesterone concentration from days 4 to 7 post ovulation of Nelore cows synchronized for ovulation and submitted to different gonadotropins treatments.

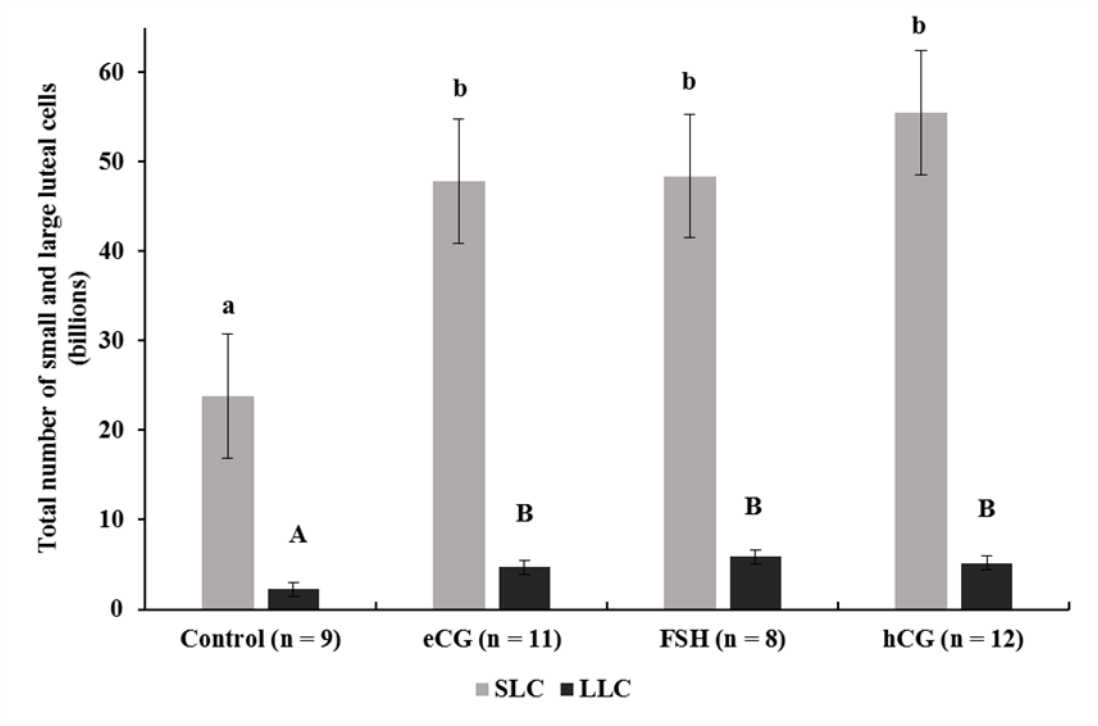

Figure 4. Least squares means ± SEM of the total number of small and large luteal cells in Nelore cows synchronized for ovulation and submitted to different gonadotropin treatments. $^{a,b}P < 0.05$ for small luteal cells; $^{A,B}P < 0.05$ for large luteal cells.

4.4 Discussion

The aim of this study was to evaluate the effect of eCG or multiple treatments of FSH or hCG on dominant follicle development, E2 concentration as well as CL volume and P4 concentration. Our first hypothesis that treatments with eCG, FSH or hCG would enhance final follicle growth leading to a greater ovulatory follicle size and circulating E2 peak was partially confirmed. Before starting the treatments, follicle size and growth rate were similar among treatments. As soon as cows received the gonadotropin treatments, they presented an overall growth rate (between D7-10; Table 2) greater than Control and larger ovulatory follicles (Table 1). Despite that, we were not able to detect a difference between treatments for circulating E2 (Figure 2). It is well known that treatment with eCG, near the time of P4 implant removal, increased follicle growth rate and ovulatory follicle diameter in cows and heifers submitted to FTAI protocols [6-8,32]. Thus, our results are in agreement with the literature and these findings are probably due to the long half-life of eCG and the dual FSH

and LH-like activity of the molecule [9]. Moreover, similar results were found for FSH, however the use of this hormone to increase follicle growth has been described to a much lesser extent. Sales et al. [6] reported that only one treatment with 10 mg FSH at the time of P4 device removal failed to stimulate follicle growth or improve pregnancy rate compared to eCG. Also, P/AI was not improved in anovular *Bos indicus* [33,34] or *Bos taurus* [16] cows after treatment with 20 mg FSH at P4 device removal in FTAI protocols. In high-producing dairy cows, Giordano et al. [21], using 80 mg of FSH reported similar results, in which FSH failed to increase follicle growth compared to Control. After follicular deviation, there is less dependence on FSH and a greater need for LH for continuous growth of the dominant follicle. Despite this fact, the level of FSH receptor mRNA and the affinity of granulosa cells to bind FSH did not vary significantly according to follicular size [35,36], and at least some circulating concentration of FSH after deviation is necessary for continued growth of the dominant follicle [2]. The lack of positive effect on these studies seem to be due to the short half-life and fast clearance of FSH, which is estimated at 5 hours and 10 to 12 hours, respectively [18]. This short half-life of FSH was the greatest reason for multiple treatments in our study, and we successfully increased follicle growth rate (Table 2) and ovulatory follicle size (Table 1), confirming the hypothesis that FSH can stimulate growth of the dominant follicle. However, the number of treatments and dose need to be revised, because FSH-treated cows presented a higher multiple ovulation rate (Table 3).

For the best of our knowledge, only one study tested the effect of hCG as stimulator of follicle growth. Prata et al. (in press), reported that treatment with 300 IU of hCG at P4 device removal induced the same growth rate as 300 IU of eCG and it was greater than two other doses of hCG (200 IU i.m. and 200 IU s.c.) and Control. However, 300 IU of hCG caused premature ovulation in 44.4% of the cows. Thus, this dose of hCG stimulated a faster follicle growth, however it was not low enough to prevent premature ovulation in dominant follicles. As shown in Tables 1 and 2, the follicular growth rate and ovulatory follicle size in hCG-treated cows were greater than Control, supporting our hypothesis. One of the major characteristics of the dominant follicle is the presence of LH receptors in the granulosa cells [35,37-39]. Furthermore, hCG binds to LH receptor in granulosa and theca cells with high affinity [19,20] and probably stimulates follicular growth. Unexpectedly, three cows on this group (12%; Table 3) presented premature ovulation even in small doses, and the most logical reason for that is the size of the follicle. All three cows had follicles > 10 mm at the time of the first hCG treatment.

When we analyzed all cows, with or without ovulation, hCG group presented greater maximal size of the dominant follicle compared with the other groups and the major reason for that and for no difference in the other groups was that cows on hCG and Control groups needed more time to reach the maximal size than eCG- or FSH-treated cows (Table 1).

Despite all the differences in ovulatory follicle diameter and maximal size of the dominant follicle, no differences were observed for circulating E2 peak concentration among treatments. Estradiol is synthetized in granulosa cells through cytochrome P450 aromatase complex and by the enzyme 17β-hydroxysteroid dehydrogenase. Furthermore, some studies reported that eCG increases E2 production by increasing the amount of mRNA for cytochrome P45017α [40,41], and FSH and LH could trigger this enzyme complex stimulating E2 production and follicle development [42-44]. These different results between our study and the literature is probably due to the different techniques, because E2 was measured in follicular fluid or media cultured with granulosa cells. In our study, we measured circulating concentrations. It is well known that many factors can interfere in circulating E2. Another plausible reason could be a dysfunction of the ovulatory follicle caused by the LH action present in hCG- or FSH-based commercial products having above 15% contamination with LH [45]. In early stages of ovulation or follicle luteinization, reduction in circulating E2 concentration was reported [46-48].

The ovulatory follicle size is well correlated with the respective CL size formed in the subsequent diestrus [49,50] and other studies also indicated that CL size is related with P4 concentrations [51-53]. However, our hypothesis that larger follicles resulted in greater CL volume and P4 concentration was refuted. Interestingly, all gonadotropin treatments presented greater number of small and large luteal cells (Figure 4), similar to the results reported by Rigoglio et al. [12] in *Bos indicus* cows treated with eCG. Large luteal cells are responsible for nearly 80% of luteal P4 (reviewed by Diaz et al [54]), and increases in circulating P4 during diestrus have been related with large luteal cells production during CL lifespan [55]. That way, the lack of difference in our study could be explained by the period that we collected the blood, between days 4 to 7 post-ovulation. It may have been too early to detect any difference between treatments. Moreover, differences in circulating P4 concentrations in studies in which cows were treated with eCG during FTAI protocols, were only detected in the middle of diestrus, 12 to 14 days after ovulation [4,56,57], suggesting that we might have detected differences in circulating P4 if we had continued to collect samples later in the cycle.

4.5 Conclusions

In conclusion, each of the gonadotropin treatments, eCG, FSH, or hCG, were effective in increasing the follicular growth rate between D7-10 and consequently the follicular diameter on D10 and ovulatory follicle diameter. In addition, treatment with different gonadotropins increased the number of large and small luteal cells, however, there was no difference in E2 peak concentration, CL volume and circulating P4 concentration post-ovulation. Therefore, this study presents other potential strategies for stimulating follicle growth in cows treated for FTAI, besides eCG.

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5. FINAL CONSIDERATION

Fixed-time AI programs are largely used worldwide, in beef and in dairy cattle. Since the first protocol (Ovsynch) was developed, many modifications were performed during the last years, generating a variety of protocols to be used in different situations with a combination of several hormones. In Brazil, the use and benefits of eCG in beef cattle is well reported in the literature, however, in crossbred dairy cows no studies were performed. That way, this study showed that eCG is an important tool to improve P/AI in grazing crossbred dairy cows submitted to FTAI with ≤70 DIM. Moreover, our study demonstrated that the use of eCG on D7 do not increase twining rate. Trying to find an alternative hormone to eCG in beef cows, we tested the hCG as an follicle growth stimulator. The results clearly suggest that a single administration of hCG on D8 does not appear to be a reliable alternative to eCG treatment in FTAI protocols, even in small doses, due to high percentage of premature ovulation or no improvement on follicle growth rate. The hCG binds to LH receptors on theca and granulosa cells with high affinity, and probably leading to follicle ovulation. However, multiple treatments with hCG or FSH presented similar results to eCG, as they were effective in increasing follicular growth rate between D7-10 and, consequently, the follicular diameter on D10, and ovulatory follicle diameter. In addition, treatment with different gonadotropins increased the number of large and small luteal cells, probably due to the larger ovulatory follicle, that may present more granulosa and theca cells. In conclusion, this study identified some physiological mechanisms of different gonadotropins in the development of the the dominante follicle, and this is the first step to develop or introduce new drugs to stimulate follicle growth like eCG in FTAI protocols.