OVARIAN CHARACTERISTICS, SERUM CONCENTRATIONS, AND FERTILITY IN LACTATING DAIRY COWS IN RESPONSE TO EQUINE CHORIONIC GONADOTROPIN

by

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Abstract

The objectives were to evaluate the effects of equine chorionic gonadotropin (eCG) administration on preovulatory follicle diameter, serum estradiol and progesterone concentration, corpus luteum (CL) diameter, estrual activity, and pregnancy rate. Lactating dairy cows were submitted to a Presynch-Ovsynch timed artificial insemination (TAI) protocol. Cows (n = 121) in a single herd were treated with 2 injections of prostaglandin $F_{2\alpha}$ (PGF) 14 d apart (Presynch), with the second injection administered 11 d before the onset of a timed AI protocol (Ovsynch; injection of GnRH 7 d before and 56 h after $PGF_{2\alpha}$, with TAI administered 16 to 18 h after the second GnRH injection). Cows were assigned randomly to receive either saline or 400 IU eCG concurrent with the $PGF_{2\alpha}$ injection of the Ovsynch protocol (d 0). Blood samples were collected during the study to monitor serum changes in progesterone and estradiol to determine if eCG would facilitate increased estrual activity, improved ovulatory response to GnRH, and enhanced post-ovulatory luteal function. Administration of eCG tended to increase the number of CL and on d 9 and 16 after $PGF_{2\alpha}$, corresponding to d 6 and 13 post-ovulation. Volume of the post-eCG treatment luteal tissue was increased only on d 16. Timed AI pregnancy rates did not differ between eCG (36.9%) and control cows (41.8%). We concluded that use of eCG provided no profertility advantages to dairy cattle when programmed for a timed insemination at first service.

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CHAPTER 1 - Introduction

The increasing world population requires efficient beef and dairy management practices to meet the demands of the consumer. A primary objective of any herdsman is to get cows pregnant as quickly as possible post-calving (Lucy et al., 2004). Reproductive performance in lactating dairy cows has declined slowly as milk production has increased during the past 20 to 30 yr (Lucy, 2001). Infertility of dairy cows in the US is a multifaceted predicament that has resulted from a combination of factors during the last decade (Lucy, 2001). During this time, milk production has increased by approximately 20% and reproductive performance has deteriorated concurrently (Lucy, 2001). Reduced conception rates and poor reproductive performance has caused an increased demand for new tools and methods to manage reproduction (Lucy et al., 2004). Failure to detect estrus and misidentification of estrus is a problem that is compounded by decreased expression of estrus because of increased utilization of concrete floors in modern dairies (Lucy, 2001). One method to increase the number of cows inseminated in a short period is to apply timed artificial insemination (**TAI**) protocols that synchronize ovulation without a requisite period of detecting estrus (Bó et al., 2007). The objective of this review is to present recent data from studies in which contemporary protocols were utilized with the addition of equine chorionic gonadotropin (**eCG**) administration, and its effects on fertility outcomes in the bovine.

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CHAPTER 2 - Equine Chorionic Gonadotropin (eCG)

In 1930, Cole and Hart reported a substance found in the serum of pregnant mares collected between 37 and 131 d of gestation that induced pronounced ovarian growth response in immature rats. This substance was called pregnant mare serum gonadotropin (PMSG), and now is appropriately termed eCG because it is of chorionic origin (Murphy and Martinuk, 1991), which is more consistent with other chorionic gonadotropins (**CG**) produced during pregnancy in primates. It is synthesized from the chorionic girdle cells initially, and later, from the endometrial cups cells (Thway and Wolfe, 2001). Unique to eCG is its ability to elicit both FSH- and LH-like responses in non-equine mammals (Licht et al., 1979). Equine CG has a longer half-life than FSH or hCG because of its glycosylation, which has practical application in its prolonged physiological activity (Matsui et al., 1994). The FSH-like properties of eCG and its availability have led to its application as a convenient exogenous hormone used to stimulate follicular growth and superovulation in laboratory and farm animal species (Hoppen, 1994).

Structure of eCG

Thyroid stimulating hormone (**TSH**), FSH, LH, human CG (**hCG**), and eCG are all members of the same glycoprotein family (Thway and Wolfe, 2001). This glycoprotein hormone family is characterized by a heterodimeric structure composed of a noncovalently bonded α-subunit and a species-specific β-subunit (Papkoff and Samy, 1967; Pierce and Parsons, 1981). Only the heterodimers are biologically active and not the subunits (Pierce and Parsons, 1981). The α-subunit is not only essentially identical between the 5 hormones, but is highly conserved across species (Pierce and Parsons, 1981). The β-subunit demonstrates homology in structure, but

unique differences occur in carbohydrate compositions among species (Pierce, 1971). Thus, the β-subunit dictates its hormone action and species specificity regardless of the α-subunit to which it is bound (Pierce, 1971). The carbohydrate moiety of the gonadotropins is essential for evoking the subunit association, biological activity, correct protein folding conformation, secretion, and half-life (Hartree and Renwick, 1992). The glycoprotein hormones elicit a biological response once bound to their respective receptor via the stimulation of cyclic adenosine monophosphate (**cAMP**) synthesis followed by subsequent effects on activity of assorted protein kinases (Pierce and Parsons, 1981).

Primary Structure

The α-subunit of eCG or of the glycoprotein hormone family consists of 96 amino acids with a molecular weight of 17 kDa, and the β-subunit 149 amino acids with a molecular weight of 44 kDa (Pierce and Parsons, 1981; Sugino et al., 1987; Hokke et al., 1994). Each subunit is composed of a peptidic part coupled to a glycan moiety (*N*- and *O*-chains; Murphy and Martinuk, 1991). The α-subunit is highly conserved and common to all glycoproteins because it is the product of a single gene (Boothby et al., 1981; Stewart et al., 1987). The β-subunit is specific and differs in its amino acid sequence among the glycoprotein hormones, thus the β-subunit is the hormone determinate (Combarnous, 1988). In the case of eCG, the polypeptide chain of amino acids is identical to the pituitary hormone, eLH, except for its carbohydrate content (Legardinier et al., 2005). The β-subunit differences in glycosylation are the source of eCG's unique biological activities and are responsible for its receptor binding specificity (Pierce, 1971; Pierce and Parsons, 1981). It has recently been shown that the sequence of amino acids 104 to 109 of the β-subunit is an important region for its efficient secretion and full FSH activity (Galet et al., 2009).

Secondary Structure

The α-subunit of eCG has 10 half-cystine residues or 5 disulfide bridges, whereas the β-subunit contains 12 residues or 6 bridges, which is similar to all known glycoproteins (Combarnous, 1988; Murphy and Martinuk, 1991). Ward and Bousfield (1990) assigned disulfide bonds bridging Cys^{11} to Cys^{36} and Cys^{14} to Cys^{35} of the N-terminal portion of the α-subunit. In the C-terminal portion, disulfide bonds join Cys^{64} to Cys^{91} and Cys^{87} to Cys^{89}; in the core Cys^{32} interact with Cys^{65}. The known disulfide bonds of the β-subunit have been located between the N- and C-terminal regions between Cys^{38} to ^{57} and Cys^{93} to Cys^{100}, providing the "long loop" and "determinate loop," respectively (Ward and Bousfield, 1990). Another C-terminal portion of the β-subunit contains a disulfide bond located between Cys^{26} and Cys^{110}, which participates in a "seat-belt" formation around the α-subunit (Galet et al., 2004). The sequence similarity of half-cystine residues in all β-subunits, together with their strong binding to a common α-subunit, indicates that disulfide bonds occur in corresponding positions in all β-subunits (Hartree and Renwick, 1992). The interchanges of the disulfide bonds have demonstrated their role in stabilizing the eCG heterodimer (Galet et al., 2004; Legardinier et al., 2008).

Equine CG has 30 residue carboxyl-terminal extensions in the β-subunit, unlike other members of the glycoprotein hormone family, which only recurs in hCGβ and eLHβ (Pierce and Parsons, 1981). The amino acid sequence of eCGβ is identical to that of eLHβ, which explains the observations of eCG exhibiting the same intrinsic FSH-like activity of eLH (Sherman et al., 1992). The carboxyl-terminal peptide is laden in both proline and serine residues (Pierce and Parsons, 1981). The function of the extended carboxyl-terminal end is unknown, but has been

proposed to serve an immunological role or protection from proteolysis (Pierce and Parsons, 1981).

Glycoslyation

Approximately 45% of the mass of eCG is attributed to carbohydrate moieties, which make it one of the most glycosylated glycoproteins (Papkoff, 1978; Murphy and Martinuk, 1991). The α-subunit is approximately 22% carbohydrate based on weight, and the β-subunit is about 50% (Christakos and Bahl, 1979; Damm et al., 1990). Glycosylation occurs on both α and β-subunits at asparagine- (N-glycosylation), serine-, or threonine-linked (O-linked) sites (Murphy and Martinuk, 1991). The α-subunit has 2 complex type N-linked oligosaccharide chains found at Asn^{56} and Asn^{82} (Legardinier et al., 2005). The eCGα N-linked carbohydrates tend to be smaller than those of eCGβ (Anumula and Bahl, 1983). Only one N-glycoslyation site is present on the β-subunit located at Asn^{13} (Bousfield et al., 1987; Legardinier et al., 2005). Approximately 80% of the total N-linked oligosaccharides have been elucidated on the β-subunit (Matsui et al., 1994). Equine CGβ contains an assortment of mono- to penta-antennary complex-type N-linked oligosaccharides (Matsui et al., 1994). An N-acetyllactosamine polymer has been identified among the N-linked oligosaccharides, which is unique to eCG (Hoppen, 1994). Most of the O-glycosidically linked chains have been identified as trisaccharide Neu-5Acα2-3Galβ1-3GalNAc-ol (Hoppen, 1994). The biological significance of the sugar chains is not fully understood, however, it has been suggested that the terminal residues in N-linked oligosaccharides of eCG assist in the regulation of signal transduction and the half-life of the hormone (Matsui et al., 1994).

The O-linked carbohydrate chains seem to be specific to CG's (Hoppen, 1994). Approximately 547 nmol of O-linked carbohydrate chains are contained within the β-subunit

(Damm et al., 1990). Therefore, approximately 6 O-linked glycosylation sites are located on the β-subunit (Damm et al., 1990). The β-subunit contains 12 O-glycosylation sites: Ser^{118}, Ser^{123}, Ser^{128}, Ser^{130}, Ser^{137}, Ser^{140}, Ser^{141}, Ser^{149}, Thr^{127}, Thr^{129}, Thr^{131}, and Thr^{133} (Bousfield et al., 2001). The O-linked oligosaccharides have been accredited for the prolonged circulatory survival of eCG (Galet et al., 2009).

The most prominent aspect of eCG glycosylation is the exceptionally high quality and quantity of sialic acid content (Hoppen, 1994). The main sialic acid constituents of eCG are α2-6-linked Neu5Ac resides, which are rarely found in other glycoprotein hormones (Hoppen, 1994). Exclusive to eCG is the O-acetylation of sialic acid (Hoppen, 1994). The sialic acid chains have been accredited for the long half-life of eCG (Hoppen, 1994).

Tertiary structure

Initial information of the tertiary structure of eCG is given by the position of the disulfide bridges (Combarnous, 1988). Disulfide bonds seem to be one of the major forces in the stability of eCG's tertiary structure (Pierce and Parsons, 1981). Because of commonality of the half-cystine residues in all the glycoprotein hormones, which occupy identical positions and sequence, it is generally accepted that their disulfide bridges are also similar (Combarnous, 1988). Without accurate determination of all the disulfide bond placements within eCGβ, the three-dimensional model or x-ray crystallography of the complete structure has yet to be achieved (Pierce and Parsons, 1981).

Quaternary structure

Equine CG shows evidence of a complex quaternary structure wherein the α-subunit is secured by the C-terminal extension or "seatbelt" loop of the β-subunit, which involves the disulfide bridge between Cys^{26} and Cys^{110} (Galet et al., 2004). The association of the 2 subunits

requires noncovalent bonding and cooperative folding for biological activity (Galet et al., 2004). The eCG heterodimer is protected by the bulky carbohydrate chains, which protects the fragile disulfide bridge of the seatbelt" loop (Galet et al., 2004). Increased stability of the quaternary structure of eCG compared with eLH results from the protection and stabilization of the Cys^{26} and Cys^{110} disulfide bridge (Galet et al., 2004). The manner in which the carbohydrate chains stabilize the disulfide bridge is not known (Galet et al., 2004).

Biosynthesis of Equine Chorionic Gonadotropin

Each subunit is biosynthesized by the complex process established for other hormones in the glycoprotein family (Hartree and Renwick, 1992). The α- and β-subunit is encoded by a single copy gene. The α gene is found in both the pituitary and placenta, whereas the β gene is only encoded in the placenta (Pierce and Parsons, 1981). The genetic information is transcribed and translated by the mRNA (Hartree and Renwick, 1992). Specific post-translational modifications occur in the rough endoplasmic reticulum, including the addition of high-mannose N-linked oligosaccharides and the cleavage of the leader sequence (Hartree and Renwick, 1992). Manger and Weintraub (1982) reported the association of α- and β-subunits initiate in the rough endoplasmic reticulum, but primarily occur in the smooth endoplasmic reticulum or Golgi apparatus. The remaining stages of N-linked oligosaccharide synthesis, O-linked glycosylation, and sulfation are proposed to occur in the Golgi apparatus (Hirschberg and Snider, 1987). Multiple forms of a hormone or subunit are almost always encoded by a single gene, and the differences are a result of translational or post-translational events (Hartree and Renwick, 1992).

Estrogen has a repressive effect on expression of the α-subunit in most mammals (Gharib et al., 1990). The mare does not have this mechanism for repression (Farmerie et al., 1997). It was

later shown that eCGα mRNA levels increased in response to estrogen (Robinson et al., 1995). This represents a divergent mechanism of regulation for glycoprotein hormone biosynthesis of the α-subunit in placenta (Farmerie et al., 1997). Presently, no autocrine, endocrine, or paracrine factors have been identified to control the synthesis of eCG (Hoppen, 1994).

Secretion of eCG

Equine CG is synthesized by specialized placental trophoblast cells of the chorionic girdle as early as 32 d of gestation in the mare (Thway and Wolf, 2001). The chorionic girdle is comprised of a pale band of the trophoblast around the spherical conceptus along the line where the yolk sac and allantois adjoin (Hoppen, 1994). At approximately d 36 of gestation, endometrial cups, which are circumscribed plaques of tissue located in the gravid horn endometrium, are created from patches of chorionic girdle cells (Clegg et al., 1954; Murphy and Martinuk, 1991). The endometrial cups are formed by the invasion of the endometrium by the specialized cells of the trophoblast (Clegg et al., 1954). The mature endometrial cup becomes established in the endometrial stroma by 1 to 2 d (Hoppen, 1994). Endometrial cups take over synthesis of eCG before attachment of the allantochorion to the uterine epithelium (Ginther, 1992). Hormone production and secretion classifies the endometrial cups as a transient placental endocrine gland (Senger, 2005). Secretion of eCG and weight of the endometrial cups are highly correlated (Ginther, 1992).

The mature endometrial cup contains a cytoplasm full of mitochondria, rough endoplasmic reticulum cisternae, and Golgi apparatus (Wooding et al., 2001). Immunocytochemistry has shown that eCG is localized in the Golgi cisternae in small dense granules (Wooding et al., 2001). Therefore, release of eCG seems to occur via the customary exocytotic mechanism as established for other protein hormones (Wooding et al., 2001). Absence of significant

accumulation suggests that the endometrial cup cells secrete eCG constitutively, without secretogogues (Wooding et al., 2001). It has been concluded that eCG is secreted by the epithelium of the dilated glands within the endometrial cups (Clegg et al., 1954). Equine CG then enters the circulating bloodstream via a complex of lymph sinuses located in the endometrial stroma underneath each cup (Cole and Goss, 1943). No regulatory mechanism has been discovered to control the secretion of eCG (Hoppen, 1994).

Several factors have been reported that influence the secretion of eCG from the endometrial cups. These factors include size, breed of mare, sire, parity, and number and genotype of fetuses (Davies-Morel, 2003). The latter was discovered by an increase in eCG concentration of 203 IU/mL by a donkey carrying a hinny fetus from 120 IU/mL compared with of a mare carrying a horse fetus (Allen, 1969). Effects of sire and mare have influenced the amount of eCG secreted, but the influences have yet to be further elucidated (Murphy and Martinuk, 1991). Draft mares have lesser circulating concentrations of eCG than light breed mares (Allen, 1982). Lesser concentrations were attributed to a dilution effect of a larger blood volume in the draft mares, although this hypothesis is not supported by any evidence (Allen, 1982).

Persistence of eCG in Circulation

The overall activity of eCG is dependent on its efficiency at the different levels of its action (Combarnous, 1988). The first level is the hormone's half-life and clearance rate from circulation (Combarnous, 1988). Second is eCG's ability to recognize a specific receptor (Combarnous, 1988). The third and final level is its efficiency in triggering a response in a specific target cell (Combarnous, 1988). The structure of eCG on the 3 levels of action affects its biological efficiency in general circulation.

Half-life and Clearance Rate

Studies have indicated a 2-compartment model of disappearance of eCG from circulation (Schams et al., 1978; Murphy and Martinuk, 1991). The first component is the distribution phase, which is distinguished by the rapid removal of the glycoprotein from circulation, which was determined to be 45.6 h in the bovine and 72 h in the equine (Catchpole et al. 1935; Schams et al., 1978). Second is the elimination component, which includes the hormone's metabolism and secretion (Murphy and Martinuk, 1991). The half-life of the elimination phase was found to be 121 h in the bovine and 6 h in the equine (Catchpole et al. 1935; Cole et al., 1967; Schams et al., 1978).

Persistence of eCG in circulating blood of the mare and other species is longer than any other glycoprotein hormone because of both its glycosylation and sialic acid content (Aggarwal and Papkoff, 1981; Murphy and Martinuk, 1991). The glycosylation acts to prolong the activity of eCG in circulation by C-terminal extension of the β-subunit, which seems to extend hormonal survival (Bousfield et al., 1994). This extended half-life is in part a result of the sialic acid linkages found at the ends of biantenna glycans, which are moderately resistant to neuraminidases (Hoppen, 1994). Removal of sialic acids from eCG dramatically reduces its in vivo activity without influencing binding ability (Yang and Papkoff, 1973). Only after the sialic acids residues have been removed from eCG can the molecule be taken up by the liver via the asialoglycoprotein system (Hudgin et al., 1974; Weiss and Ashwell, 1989). It was reported that clearance of the majority of eCG in the mare was by degradation and metabolism rather than by renal filtration (Cole et al., 1967).

Receptor Binding in the Equine

In the mare, eCG binds to the LH/CG receptor and has no FSH activity (Stewart and Allen, 1981). In nonequine mammals, eCG stimulates both a FSH and LH response (Licht et al., 1979). Dual activity is not an inherent property of eCG, but results from the binding of the hormone to both LH and FSH receptors (Combarnous, 1988; Hoppen, 1994). Discovery of an antigenic area on eCG that corresponds to the interaction site with both the LH and FSH receptors may indicate that the α-subunit is primarily involved in binding and β-subunit to a lesser extent (Chopineau et al., 1993). This binding phenomenon is supported by an earlier finding, which demonstrated the binding of eCGα to FSH receptors, thus stimulating FSH activity in the rat (Aggarwal et al., 1981). The receptor and glycoprotein hormone interaction requires the carbohydrate terminal pentapeptide of the α-subunit (Pierce and Parsons, 1981). Binding and structural basis for dual hormone activity of eCG has yet to be fully elucidated (Hoppen, 1994).

Affinity of the hormone-receptor binding varies among hormones (Senger, 2005). Equine CG binds to eCG/LH receptor (R) with one tenth or less affinity of eLH (Stewart and Allen, 1981). Differences in binding activity between eCG and eLH may be a result of differences in size and structure of the carbohydrate chains linked to the polypeptides of the α- and β-subunits (Saint-Dizier et al., 2004).

Response of Target Cells

All glycoprotein hormones elicit their respective biological response after interacting with a hormone-specific receptor on the membrane of the target cell (Pierce and Parsons, 1981). A common receptor is used by both eCG and eLH in the mare (Saint-Dizer et al., 2004). Equine CG/LH-R are located in the main intracellular adenylate cyclase signaling pathway activated by the receptor, which results in an increase in intracellular cAMP (Ascoli et al., 2002). The glycan

chains of eCG are involved in its stability and required for the efficiency of signal transduction (Herve et al., 2004). The hormone-receptor complex activates adenylate cyclase and G-proteins (Senger, 2005). Activated adenylate cyclase converts adenosine triphosphate (ATP) to cAMP (Senger, 2005). Cyclic AMP then activates protein kinases, which are a cluster of enzymes located in the cytoplasm that consist of a regulatory and catalytic subunit (Senger, 2005). Protein kinases activate additional enzymes that convert substrates into products via binding of the regulatory subunit to cAMP, and then the catalytic subunit activates enzymes that convert existing substrates into estradiol or progesterone (Senger, 2005).

Physiological Effects of eCG in the Equine

The biological role of eCG in the mare has been a matter of discussion. Proposed functions have included: maintenance of pregnancy, a relic of evolution, induction and support of primary corpus luteum (**CL**), stimulation of fetal gonads, and regulation of the immune system (Hoppen, 1994). Currently, it is accepted that the functions of eCG in the mare are to preserve the primary CL and to form secondary or accessory CLs in order to maintain the pregnancy (Senger, 2005). Equine CG stimulates CL growth and increases production of progesterone by the primary CL (Saint-Dizier et al., 2003). This resurgence of the primary CL is distinguished by a decrease in progesterone production from d 28 to 30, then an increase in expression of steroidogenic enzymes coincident with the secretion of eCG (Davies-Morel, 2003; Saint-Dizier et al., 2003). Between d 40 and 70, eCG induces ovulation or luteinization of follicles >30 mm by binding to eLH-receptors (Davies-Morel, 2003; Saint-Dizier et al., 2004). Additional CLs contribute to the secretion of progesterone as a supplementary source of steroids (Saint-Dizier et al., 2003). Equine CG and subsequently formed accessory CLs may serve as a redundant system to ensure the maintenance of pregnancy until placental structures assume the responsibility as the primary

steroidogenic endocrine gland of pregnancy (Murphy and Martinuk, 1991). Regression of all CL occurs once luteotropic stimuli of eCG is no longer available at approximately d 100, because of gradual transition from an ovarian to placental source of progesterone (Saint-Dizier et al., 2003). A localized maternal cell-mediated immunological reaction to paternal antigens in the fetal chorionic cells results in the demise of the cup cells and termination eCG synthesis and subsequent secretion (Allen and Moor, 1972; Hoppen, 1994).

Receptor Binding in the Bovine

Numbers of FSH receptors are greatest in maturing follicles on d 4 of the first follicular wave of the bovine estrous cycle when LH receptors are initially detected in granulosa cells of the dominant follicle (Xu et al., 1995). After having acquired LH receptors, dominant follicles respond to both LH and FSH or eCG (Ireland and Roche, 1982). Binding of eCG to FSH or LH receptors primarily occurs by interaction of the α-subunit (Chopineau et al., 1993). The effects of second messengers, such as cAMP, are not dependent on the hormone that leads to their production (Combarnous et al., 1988). Therefore, eCG binding to the FSH receptor stimulates a transmembrane signal transduction resulting in elevated levels of cAMP by activation of adenylate cyclase (Combarnous et al., 1988). An increased level of cAMP activates protein kinase A (PKA), which stimulates the steroidogenic pathway for production of estradiol-17β by dominant follicles (Xu et al., 1995).

Profertility Effects of eCG in the Bovine

The dual LH and FSH activities of eCG, its longer half-life, and availability in large quantities make it a unique and convenient exogenous gonadotropin to induce ovulation and stimulate follicular growth in laboratory and farm animals (Hoppen, 1994). Equine CG may be used to enhance follicular growth (Maraña et al., 2006; Sá Filho et al., 2010a), induce ovulation

(Baruselli et al., 2004; Sá Filho et al., 2004; 2005; 2010a), increase function (Bergmaschi et al., 2005; Sá Filho et al., 2010b) and size of the subsequent CL (Sá Filho et al., 2005; Souza et al., 2006; Sá Filho et al., 2010b), and increase pregnancy rates (Baruselli et al., 2003; 2004a; Bó et al., 2007). Effects of eCG have been shown to enhance fertility and reproductive efficiency in beef and dairy cattle.

Increased Signs of Estrus

Estrual behavior is induced by elevated estradiol in the absence of progesterone (Senger, 2005). Estrual behavior in the cow is determined by female-female mounting interactions, in which the female standing to be mounted is considered to be in estrus (Senger, 2005). Heifers and cows visually detected in standing estrus have greater follicle diameter, increased circulating estradiol concentrations, and increased pregnancy rates compared with cohorts not showing estrus (Perry et al., 2005; 2007). Cows exhibiting standing estrus may have achieved sufficient estradiol concentrations required to prepare follicular cells for luteinization (Perry et al., 2005). Cows displaying standing estrus have greater estradiol concentrations, which are necessary for the preovulatory LH surge, increased myometrial tone, and vaginal mucosal secretions (Senger, 2005).

Perea et al. (2008) evaluated the effects of hormonal and biostimulation protocols on calving to conception intervals compared with a control. Crossbred (*Bos taurus* x *Bos indicus*) anestrous cows were treated with a hormonal protocol, which included 250 mg medroxyprogesterone acetate (**MAP**) via an intravaginal sponge and injections of MAP, estradiol, and eCG. A bio-stimulation treatment included calf-removal for 120 h from d -5 to 0, and a control. Estrus was detected visually for 1 h each morning and evening with the aid of altered bulls. The hormonal

treatment increased the percentage of cows showing estrus during 30 d post-treatment by 55.3 and 26.2% compared with cows in the control and biostimulation treatment, respectively.

In contrast, Duffy et al. (2004) evaluated the effect of eCG or estradiol benzoate (**EB**) on estrous response when administration occurred pre- or post-selection of the dominant follicle (Duffy et al., 2004). Twenty-eight postpartum lactating anestrous beef cows received either eCG or EB i.m. after norgestomet ear implant removal. A large percentage of cows (26 of 27) exhibited estrous activity in response to both eCG and EB; therefore, treatment with eCG did not affect the number of cows detected in estrus.

Similar to the results reported by Duffy et al. (2004), Souza et al. (2009) reported that eCG had no effect on estrous activity. Lactating Holstein cows were assigned randomly to receive $PGF_{2\alpha}$ i.m. at the removal of an intravaginal controlled internal drug release (CIDR) insert containing progesterone and either eCG, EB injections, or both. Cows were monitored for standing estrus during 48 h post-CIDR withdrawal determined by a pressure sensitive heat mount detectors (Kamar). Estrous activity was minimal with only 18.8% of cows exhibiting standing estrus and neither eCG nor EB had an effect, regardless of ovulatory stimulus used.

Reports of increased estrous activity by eCG administration are inconsistent. Of the 3 papers monitoring estrous activity in response to eCG, only Perea et al. (2008) found an increase in estrous activity in anestrous lactating beef cows when compared with temporary calf-removal or control. The remaining 2 reports found no effect on estrous activity by treatment with eCG in anestrous lactating beef (Duffy et al., 2004) or lactating dairy cows (Souza et al., 2009). The experiment performed by Duffy et al. (2004) reported a greater proportion of cows exhibiting estrus than that reported by Souza et al. (2009). Anestrous lactating beef cows showed more

estrous activity, whereas lactating dairy cows demonstrated less activity regardless of eCG administration.

Increased Follicle Diameter in the Bovine

The diameter of the preovulatory follicle may be used as an indicator of the follicle's competence, physiological maturity, and steroidogenic capacity. The preovulatory follicle is one that has obtained dominance through the recruitment and selection phases (Senger, 2005). As the dominant or preovulatory follicle increases in size, it synthesizes increasing amounts of estradiol (Senger, 2005). Once the estradiol concentration reaches a threshold level, it activates the preovulatory LH surge (Senger, 2005). McNatty et al. (1979) found that increased follicular diameter correlated with increased follicular fluid estradiol concentrations compared with follicles of lesser diameter. Mature preovulatory follicles have sufficient granulosa cells, LH-R on granulosa and theca cells, and granulosa cells able to synthesize adequate progesterone post-luteinization (Perry et al., 2005). Diameter of the preovulatory or dominant follicle may be used as a strong indicator of follicle maturity for both *Bos taurus* (Perry et al., 2005; 2007) and *Bos indicus* (Dias et al., 2009; Meneghetti et al., 2009) cattle. This relationship between the diameter of the preovulatory follicle and increased fertility has been observed in heifers (Sá Filho et al., 2008a; Dias et al., 2009; Meneghetti et al., 2009) and lactating cows (Sá Filho et al., 2008b). Protocols that include eCG increase the size and physiological maturity of the preovulatory follicle, which may subsequently enhance fertility in heifers and cows (Perry et al., 2007).

Exogenous administration of eCG at the time of norgestomet ear implant removal and $PGF_{2\alpha}$ injection has been shown to increase the diameter of the dominant follicle before TAI. Nellore (*Bos indicus*) heifers were treated with a TAI protocol including a norgestomet ear implant, EB, and either 0 or 400 IU eCG (Sá Filho et al., 2010b). Treatment with eCG increased the rate of

dominant follicle growth from implant removal to insemination. The heifers receiving eCG had dominant follicles of greater diameter at TAI than non-treated heifers. A similar experiment was performed in Nellore heifers (Sá Filho et al., 2005) where CL determination occurred on d 0 and 400 IU eCG was administered on d 8. Heifers receiving eCG had dominant follicles that tended to have a greater diameter on d 8 compared with control heifers with CLs on d 0.

In 2 experiments, 50 anestrous lactating Nellore cows were treated with norgestomet implants with or without eCG and GnRH. Sá Filho et al. (2010a) classified cows as anestrus after 3 ovarian ultrasound examinations 7 d apart failed to detect a CL. Equine CG increased diameter of the dominant follicle on d 11 and increased the rate of growth of the preovulatory follicle from d 9 to 11.

Treatment with eCG increased growth rate of the ovulatory follicle in lactating multiparous cows compared with controls (Maraña et al., 2006). Thirty-nine crossbred *Bos indicus* cows were submitted to a TAI protocol including treatments with a progesterone intravaginal insert (DIB), EB, with or without 400 IU eCG, temporary calf weaning, or both. Diameter of the dominant follicle did not differ among treatments. Rate of growth of the preovulatory follicle was increased in cows receiving eCG compared with those that did not, regardless of temporary calf removal.

Lactating Nellore were submitted to TAI following a treatment protocol including a CIDR insert, EB, and either eCG or no eCG (Baruselli et al., 2003). Cows receiving eCG had more follicles ≥8 mm in diameter compared with those of the control. Treatment with eCG seemed to have a greater effect in cows with a more pronounced anestrus condition indicated by the presence of only small (>8 mm) follicles.

A study by Bó et al. (2007) was comprised of 4 experiments to evaluate the effect of eCG added to 2 TAI protocols. Experiment 1 treated 40 lactating Holsteins using 4 treatments to determine the effects on emergence of the follicular wave and the ovulatory follicle. The cows received a TAI protocol involving an intravaginal progesterone insert, either EB or GnRH, and either 0 or 400 IU eCG at $PGF_{2\alpha}$ administration (Figure 2.1). Emergence of the follicular wave was delayed in cows receiving EB compared with those receiving GnRH. Treatment with eCG did not increase the diameter of the preovulatory follicle.

Effect of administering eCG simultaneous with progesterone insert withdrawal on follicular growth, ovulation, and plasma progesterone concentrations was evaluated by Marques et al. (2003). Fifty cross-bred suckled primiparous (*Bos indicus*) cows received either 0 or 400 IU simultaneously with progesterone insert removal. Treatment with eCG did not affect the diameter of the dominant follicle compared with the control.

The findings of Sá Filho et al. (2004) contrast with those previously reported. Fifty lactating Nellore (*Bos indicus*) cows were classified as anestrous after 2 ultrasound examinations on d -14 and -7 before initiation of treatment. The same protocol was used as that reported by Sá Filho et al. (2010a). Diameter of the ovulatory follicle was not affected 3 d post-eCG treatment, which was determined by ultrasound examinations every 12 h from d -1 until d 3 relative to eCG administration (eCG:1.30 ± 0.06 vs. no eCG:1.28 ± 0.08 mm).

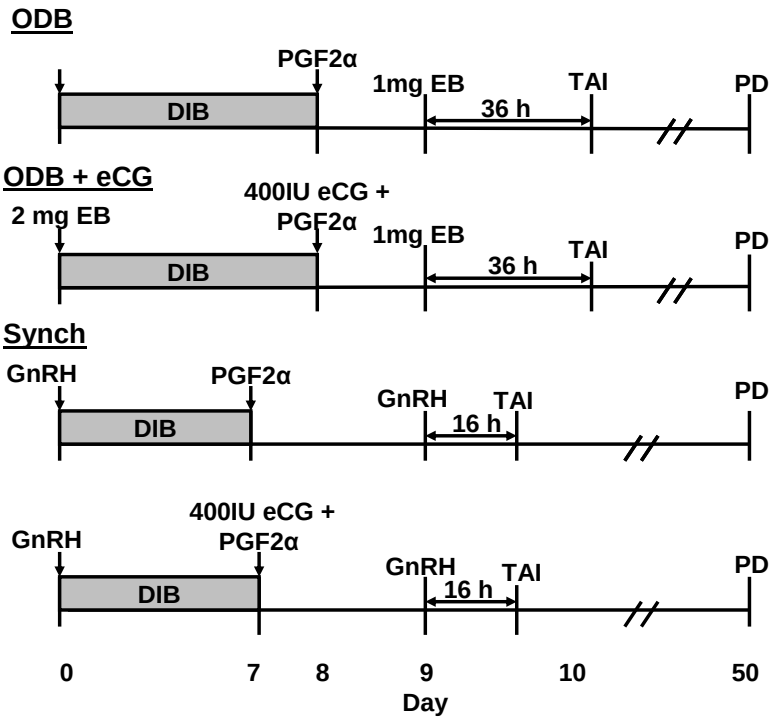


Figure 2.1 Experimental protocol as described by Veneranda et al. (2006). EB = Estradiol benzoate; DIB = Intravaginal progesterone-releasing device; PD =Pregnancy diagnosis determined by rectal palpation 50 d post-TAI.

Effects of ovulation synchronization treatments following TAI in lactating Holstein cows were evaluated by Veneranda et al. (2006). No treatment effect was detected for the diameter of the dominant follicle on d 8. At ovulation, eCG did not increase the diameter of the ovulatory follicle.

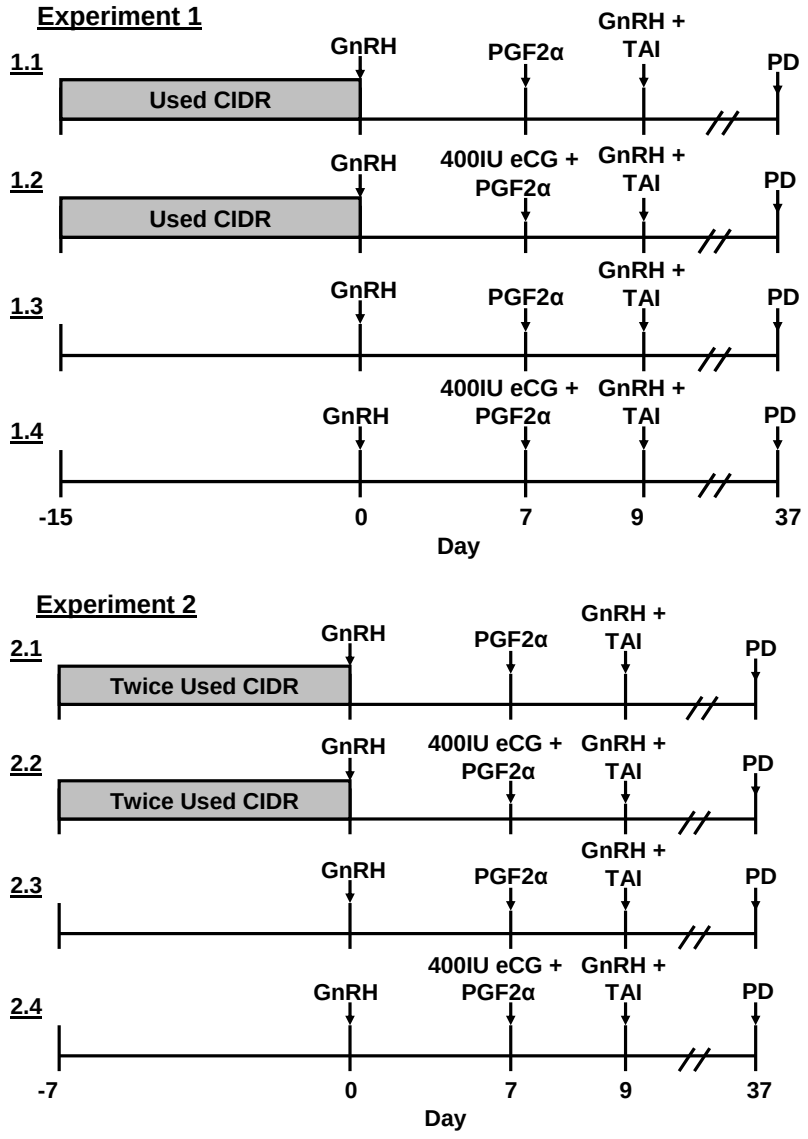


Figure 2.2 Experimental protocol adapted from Small et al. (2009). Used CIDR = Controlled internal drug release device, which originally contained 1.9 g progesterone, that had been previously used once or twice and autoclaved between uses; PD = Pregnancy diagnosis by ultrasonography on d 37.

Two experiments were conducted by Small et al. (2009) to determine the effects of low-dose progesterone and eCG to GnRH-based TAI in beef (*Bos taurus*) cattle failed to find an increase in the diameter of the preovulatory follicle. In Experiment 1, 292 suckled beef cows were

assigned randomly to 4 treatments (Figure 2.2). Treatment with eCG had no effect on the diameter of the preovulatory follicle in Experiment 1. Experiment 2 involved 141 suckled beef cows assigned by parity and calving date to 4 treatments (Figure 2.2). The diameter of the preovulatory follicle was not affected by the eCG treatment. The authors concluded that the presynchronization treatment had already increased the diameter of the preovulatory follicle; therefore, eCG had no additive effect on follicle diameter.

It was reported by Bó et al. (2007) that eCG treatment did not influence the dominant follicle diameter following progesterone insert removal. Forty Holstein cows were blocked by days postpartum and assigned randomly to 4 treatments. Cows in the progesterone and estradiol treatment (P4 + OBD) received an intravaginal progesterone-releasing insert (DIB) and EB i.m. on d 0. Eight days later, the inserts were removed and cows were administered $PGF_{2\alpha}$. Cows were then allotted to receive either 400 IU eCG i.m. or no eCG on d 8. Cows in progesterone and synchronization group (P4-Synch) received an intravaginal progesterone-releasing insert (DIB) and GnRH on d 0. Seven days later, inserts were removed; cows received $PGF_{2\alpha}$ and either 400 IU eCG or no further treatment. Twenty-four hours later, all cows were treated with EB i.m and TAI was performed 16 h later. Treatment with eCG had no effect on the diameter of the ovulatory follicle in lactating Holstein cows.

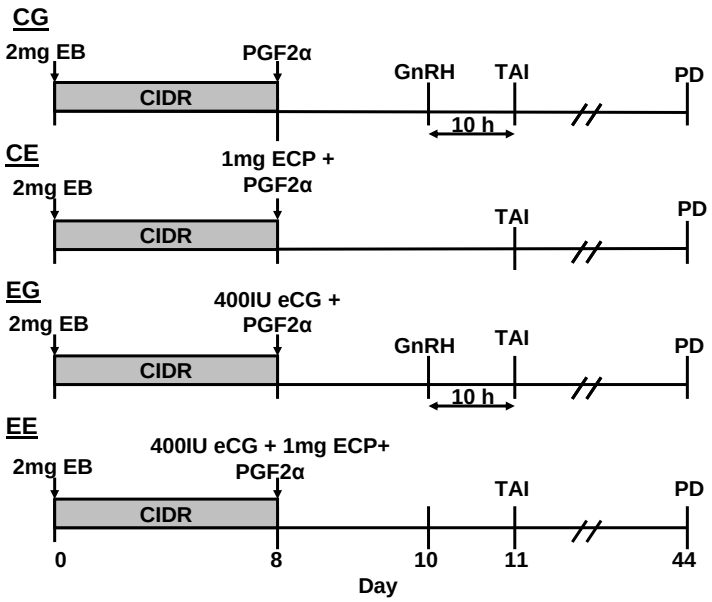


Figure 2.3 Experimental protocol adapted from Souza et al. (2009). EB = Estradiol benzoate; Controlled internal drug release device containing 1.9 g progesterone; ECP = Estradiol cypionate; PD = Pregnancy diagnosis was performed by ultrasound 33 d after TAI.

Lactating Holstein were used by Souza et al. (2009) to determine if eCG treatment and various ovulatory stimuli would increase fertility. Cows were submitted to a TAI protocol in which all cows received a CIDR and estradiol cypionate (**ECP**), which is a long-acting conjugated estradiol (Figure 2.3). Ultrasound examinations of the ovaries 48 h post-CIDR removal showed that treatment with eCG did not increase the size of the dominant follicle.

In summary, reports are conflicting with regard to whether or not treatment with eCG increases the diameter of the preovulatory follicle. Both Bó et al. (2007) and Souza et al. (2009) failed to find that treatment with eCG increased the diameter of the dominant follicle in lactating Holstein cows. Souza et al. (2009) concluded that treatment did not increase the preovulatory follicle diameter, which are in agreement with those reported in *Bos taurus* (Small et al., 2009) and *Bos indicus* (Maraña et al., 2006; Sá Filho et al., 2004) beef cows and in dairy cows (Bó et

al., 2007). In contrast, Sá Filho et al. reported that eCG treatments significantly increased the diameter of the preovulatory follicle in both anestrous suckled beef cows (2010a) and in beef heifers (2010b). Reports of increased growth of the dominant follicle have been made in both lactating (*Bos indicus*) beef cows (Baruselli et al., 2003, Maraña et al., 2006) and anestrous suckled (*Bos indicus*) beef cows (Sá Filho et al., 2010a).

Increased Ovulation Rates in the Bovine

Ovulation is a complex cascade of events that can be regulated in cattle by means of TAI protocols (Senger, 2005). Timing of ovulation is inaccurate even with TAI protocols because of variation in ovulatory response of the dominant follicle, which may occur because of differences in the physiological maturity of the pre-ovulatory follicle (Perry et al., 2005). Equine CG administration may reduce variation in ovulation timing (Cavalieri et al., 1997) and increase the incidence of ovulation (Baruselli et al., 2004a; Sá Filho et al., 2005; 2010a; 2010b) resulting in improved fertility to TAI protocols (Baruselli et al., 2004a; Sá Filho et al., 2005; 2010a; 2010b). Treatments with eCG in conjunction with current protocols may result in improved pregnancy rates by increasing ovulation rates in beef and dairy cows.

Effect of eCG on follicular dynamics and ovulation was assessed in 50 crossbred primiparous suckled cows (*Bos indicus*) by Baruselli et al. (2004). The increase in ovulation by treatment with eCG was detected in cows of lesser body condition (BCS $\leq$ 2.25). Their results indicated that eCG increased the ovulation rate by 16 percentage units compared with the control.

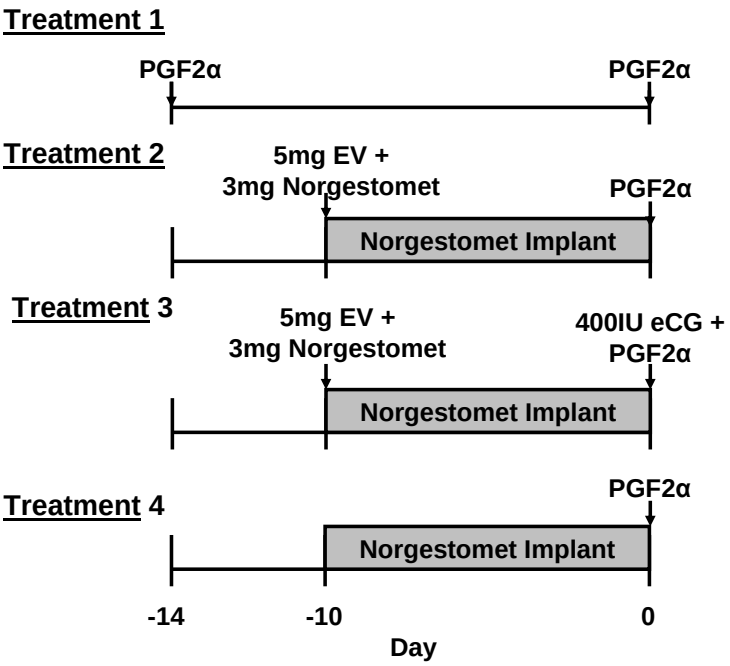


Figure 2.4 Experimental protocol adapted from Cavalieri et al. (1997). EV = Estradiol valerate; Norgestomet = 3 mg subcutaneous injection of synthetic progestogen norgestomet (17α-acetoxy-11β-methyl-19-norpreg-4-en-3,20 dione); Norgestomet implant = subcutaneous auricular implant containing 3 mg of synthetic progestogen norgestomet.

The effects of 4 estrus-synchronization protocols on ovulation were investigated by Cavalieri et al. (1997) in 48 non-lactating Brahman (*Bos indicus*) cows (Figure 2.4). All cows but one in Treatment 4 ovulated. Treatment 3, which included 400 IU eCG, significantly reduced the median interval from treatment to ovulation compared with treatments with only norgestomet-estradiol. Rate of ovulation did not differ among the 4 treatments; however, the interval from treatment to ovulation decreased in response to eCG.

Duffy et al. (2004) found that anestrous suckled cows given eCG had an increased ovulation rate. Cows that received 600 IU eCG experienced multiple ovulations. Cows given eCG during the pre-selection stage of follicle selection produced greater ovulation rates. The authors

concluded that addition of eCG to the estrus-synchronization protocol increased ovulation rate in cows treated before dominant follicle selection.

Perea et al. (2008) investigated the effects of hormonal therapy and biostimulation protocols on the reproductive response in crossbred (Brahman x Holstein) anestrous cows. Cows receiving the hormonal treatment, which included 500 IU eCG 2 d before removal of a progesterone insert, had increased in ovulation rates compared with cows assigned to the biostimulation treatment. The authors proposed that treatment with eCG may have stimulated the growth of a more functional dominant follicle, which may have been more capable of ovulation. Cows receiving eCG had increased ovulations rates compared with cows in the other 2 treatments.

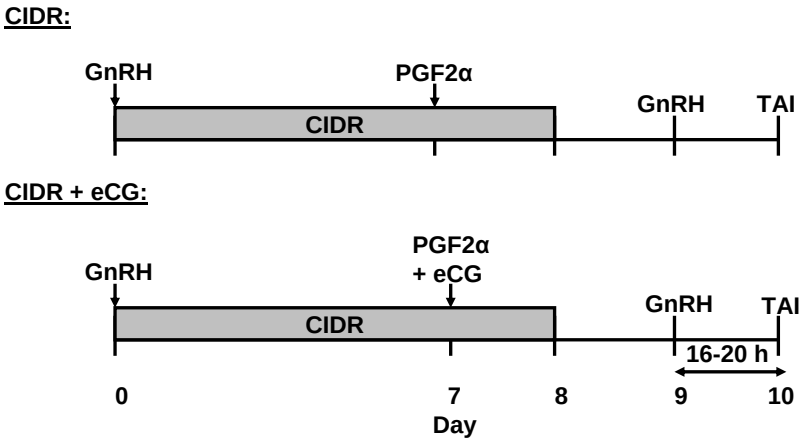


Figure 2.5 Experimental protocol for estrous synchronization adapted from Murugavel et al. (2009). CIDR = Controlled internal drug release device containing 1.38 g progesterone.

Murugavel et al. (2009) utilized lactating Murrah buffalo cows (*Bubalus bubalis*) to evaluate the effects of the addition of eCG to a progesterone-based estrus-synchronization protocol. Sixty-five multiparous lactating buffalo cows were assigned randomly to 2 treatments (Figure 2.5). Ovulation rate was determined by transrectal palpation of ovaries for absence or presence of luteal structures. Buffaloes that received eCG in addition to a CIDR had greater ovulations rates than those receiving only a CIDR. The authors proposed that administration of eCG may

have increased ovulation rates by increasing the function of the hypothalamic-pituitary-gonadal axis. Functional failure of this axis has been correlated with anestrus in buffalo cows (Saini et al., 1986). Thus, eCG administration in conjunction with exogenous progesterone supplementation supports the recovery of the hypothalamic-pituitary-gonadal axis and consequently increased ovulation rates in anestrous buffalo cows in a tropical environment.

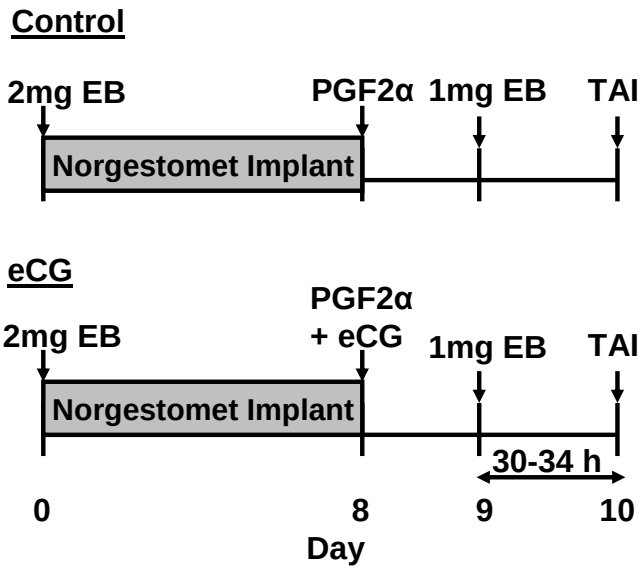


Figure 2.6 Experimental protocol as described by Sá Filho et al. (2005). Norgestomet implant = subcutaneous auricular implant containing 3 mg of synthetic progestogen; EB = Estradiol benzoate.

A series of papers by Sá Filho et al. (2004, 2005, 2010a, 2010b) reported an increased incidence of ovulation with the administration of eCG in conjunction with varied estrous synchronization protocols. Lactating anestrous beef cows that received eCG treatment had a significant increase in ovulation rate (Sá Filho et al., 2004). This effect was not seen in cows receiving no eCG or the GnRH treatments. Sá Filho et al. (2005) determined estrus-cycling status in 177 Nellore heifers before assignment to 2 treatments: eCG or control (Figure 2.6).

Ultrasonography scanning of both ovaries revealed that heifers treated with eCG had a significant increase in the rate of ovulation compared with those which did not receive treatment.

A similar protocol was used (Sá Filho et al., 2010b) as that for Sá Filho et al., (2005), with the exception of the timing of 400 IU eCG administration on d 0 rather than d 8. Administration of eCG at progestin removal increased ovulation rates compared with cows not receiving eCG treatment. In addition, when only small or medium (4 to 8 mm) sized preovulatory follicles were present at implant removal, eCG administration increased the probability of ovulation. Sá Filho et al. (2010a) utilized a protocol previously described (Sá Filho et al., 2004), in which anestrous suckled Nellore (*Bos indicus*) cows were subjected to 4 treatments. Ovulation rate was significantly increased by eCG treatment, which supported the authors' hypotheses. It was proposed by the authors that eCG treatment resulted in an increase in ovulation rates due to the anestrous physiological status of the cows used.

Most studies indicated that eCG increased ovulation rates in a high proportion of lactating cows and heifers. Addition of eCG to various estrus and ovulation synchronization protocols increased ovulation rates in anestrous: lactating buffalo (Murugavel et al., 2009), lactating beef (Duffy et al., 2004; Sá Filho et al., 2004; 2010a), and non-lactating beef cows (Perea et al., 2008). Cavalieri et al. (1997) reported eCG failed to affect ovulation rates in non-lactating cows because a large proportion of cows ovulated regardless of treatment. Conflicting results have been reported in non-lactating beef cows regarding the effect of eCG treatment (Cavalieri et al., 1997; Perea et al., 2008). Lactating dairy cows did not respond to eCG with increased ovulation rates (Bó et al., 2007; Souza et al., 2009). In contrast, 2 studies assessing the effects of eCG in beef heifers found a significant increase in ovulation rates compared with the control (Sá Filho et

al., 2005; 2010a). In summary, treatment with eCG increased ovulation rates in anestrous and/or lactating beef cows and heifers, but not in dairy cows.

Increased Luteal Activity in the Bovine

Recent experiments performed in Brazil demonstrate that treatments with eCG resulted in increased serum progesterone concentrations in the luteal phase subsequent to treatment, indicating that eCG stimulates a more competent and steroidogenic CL that may culminate in an increased pregnancy rate in lactating and non-lactating beef cows (Baruselli et al., 2004; Souza et al., 2006; Bó et al., 2007). Several authors have used exogenous eCG to increase plasma progesterone concentrations (Fuentes and de la Fuenta, 1997; Baruselli et al., 2000; 2004). Increased plasma progesterone concentrations have been associated positively with the conceptus' capacity for interferon-τ production and embryo development (Mann et al., 1999). Increasing serum progesterone concentrations by administration of eCG might negate the physiological consequences of decreased fertility resulting from heat and nutritional stress (Dobson and Smith, 2000) and decreased progesterone concentrations as a result of increased steroid metabolism in dairy cows (Sangsritavong et al., 2002; Wiltbank et al., 2006).

A positive effect of eCG was reported by Sá Filho et al. (2010b) on the diameter of the CL and circulating progesterone concentrations 5 d post-TAI in Nellore (*Bos indicus*) heifers. Transrectal ultrasonography of both ovaries was performed on d 15 of the treatment protocol to determine the presence and diameter of the CL resulting post-TAI. Diameter of the CL and circulating progesterone concentrations on d 15 were increased in response to administration of eCG. In a previous study (Figure 2.6), Sá Filho et al. (2005) also found treatment with eCG resulted in greater CL diameter 5 d post-TAI, regardless of cycling status before initiation of treatment. Treatment with eCG concurrent with norgestomet implant removal increased the

diameter of the CL post-TAI and increased progesterone in 2 studies (Sá Filho et al. 2005; 2010b).

Marques et al. (2003) reported that administration of 400 IU eCG at the time a progesterone insert was removed significantly increased plasma progesterone concentrations 12 d post-treatment. The reported increase in plasma progesterone occurred without an increase in the diameter of either the dominant follicle or the subsequent CL. Bergamashi et al. (2005) evaluated the effects of a protocol similar to that of Baruselli et al. (2003) with the administration of 400 IU eCG post-progesterone insert removal. Results showed that eCG treatment did not affect the diameter of the pre-ovulatory follicle; however, both the CL diameter and the plasma progesterone concentrations post-estrus were greater than in controls.

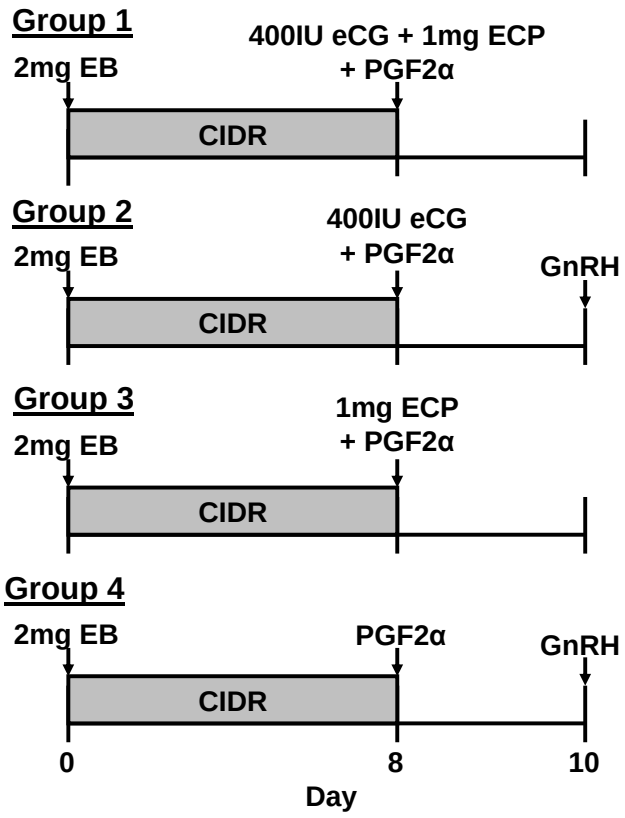


Figure 2.7 Experimental protocol as described in Souza et al. (2006). EB = Estradiol benzoate; CIDR = Controlled internal drug release device; ECP = Estradiol cypionate.

Souza et al. (2006) evaluated the effects of eCG and ECP on CL volume in Holstein cows using a TAI protocol (Figure 2.7). Treatment with eCG increased the CL volume in the diestrus period after the synchronization protocol on d 12, in addition to a tendency for increasing CL volume on d 14. The CL formed following eCG treatment was of greater volume after the synchronization protocol on d 14 than those receiving no eCG.

Souza et al. (2009) evaluated the effect of either ECP or eCG following progesterone insert removal on luteal function. Increased circulating progesterone concentration occurred 1 and 3 d post-TAI treatment in the diestrus period, which is a critical time of embryo development. The authors proposed that increasing progesterone concentrations by administration of eCG at may enhance the fertility of dairy cattle.

The physiological mechanism by which eCG stimulates increased progesterone production is unknown; however, it has been proposed that it may involve an increase in the size, ratio, or function, and duration of large luteal cells in the CL (Souza et al., 2009). Large luteal cells of the CL produce approximately 80% of progesterone. Therefore, alterations of the proportion of large to small luteal cells may be a potential mechanism for greater progesterone synthesis following eCG administration (Diaz et al., 2002; Souza et al., 2009). Treatment with eCG increased CL size in beef heifers (Sá Filho et al., 2005; 2010b), beef cows (Baruselli et al., 2003; Marques et al., 2003; Bergmaschi et al., 2005) and dairy cows (Souza et al., 2006; 2009). Increased plasma progesterone concentrations were reported by several authors (Baruselli et al., 2003; Marques et al., 2003; Bergmaschi et al., 2005; Souza et al., 2009; Sá Filho et al., 2010b) following eCG treatment. Despite a similar-sized pre-ovulatory follicles and CLs, administration of eCG increased the circulating progesterone concentrations in crossbreed lactating beef cows (Marques et al., 2003) and Holstein dairy cows (Souza et al., 2009).

Increased Pregnancy Rates in the Bovine

Timed AI protocols are advantageous in situations in which detection of estrus is difficult for various reasons in beef and dairy cows (Pursley et al., 1995). Potential problems with a TAI protocol are: 1) decreased estradiol concentrations before TAI (Souza et al., 2007a); 2) ovulation of small immature follicles (Perry et al., 2005); 3) formation of a CL of decreased diameter (Perry et al., 2005); and 4) decreased circulating progesterone concentrations (Busch et al., 2007). One solution to counter these problems is the addition of eCG to current TAI protocols. A positive correlation was reported between pregnancy rates and circulating progesterone concentrations (Bó et al., 2002; Thatcher et al., 2001; Binelli et al., 2009). Addition of eCG to existing TAI protocols may increase pregnancy rates attributable to its positive effects on several associated ovarian and uterine traits.

Treatment of anestrous Nellore (*Bos indicus*) cows with eCG at intravaginal progesterone insert withdrawal increased pregnancy rates. In a subsequent study, Baruselli et al. (2004a) reviewed 5 experiments, including Baruselli et al. (2003), to determine the effect of eCG on conception rates to TAI. Body condition scores were determined for 1,984 lactating Nellore (*Bos indicus*) cows before initiation of treatment. At removal of an intravaginal progesterone insert, cows received i.m. either 400 IU eCG or no treatment and were TAI 54 h later. Increasing pregnancy rates at 30 d post AI was dependent on the BCS before TAI protocols because only cows with poorer (≤ 3.0) BCS had improved pregnancy rates after eCG.

A study by Bó et al. (2007) summarized 4 experiments to evaluate the effect of eCG added to 2 TAI protocols. Experiment 2 assigned 394 lactating Holstein (*Bos taurus*) cows equally to the same 4 treatments used in Experiment 1. Increased pregnancy rate was attributed to the addition of eCG to the combined progesterone and EB treatment compared with other treatments.

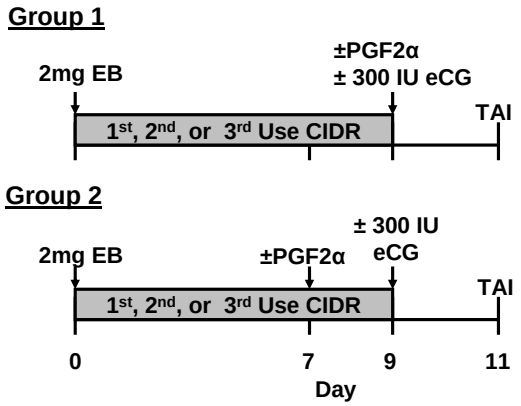


Figure 2.8 Experimental protocol as described in Dias et al. (2009). EB = Estradiol benzoate; CIDR = Progesterone-releasing intravaginal devices inserted were new (1st use), or had been used once (2nd use) or twice (3rd use) previously in a 9-d synchronization protocol.

Dias et al. (2009) evaluated in 650 cycling Nellore (*Bos indicus*) heifers the effects of altered progesterone concentrations during either a TAI synchronization protocol or after detection of estrus and AI with the addition of eCG as exogenous gonadotropic support. Heifers were assigned randomly to receive TAI on d 11 or artificially inseminated 12 h after detected estrus between d 9 and 15 (Experiment 2a and b, not shown). Pregnancy diagnoses were performed by transrectal ultrasonography 30d post-TAI. Treatment with eCG increased pregnancy rates in heifers receiving first-use CIDR, tended to increase pregnancy rates in heifers with a second-use CIDR, and had no influence on heifers receiving a third-use CIDR. The eCG treatment increased pregnancy rates in heifers receiving $PGF_{2\alpha}$ on d 9, but not when $PGF_{2\alpha}$ was administered on d 7. Treatment with eCG increased pregnancy rates; however, this effect was dependent on the timing of $PGF_{2\alpha}$ administration of new or previously used CIDR inserts.

Pregnancy rates were significantly increased in cows receiving eCG compared with those not treated with eCG (Marana et al., 2006). Authors suggested that eCG affected pregnancy rates by increasing the growth of the pre-ovulatory follicle.

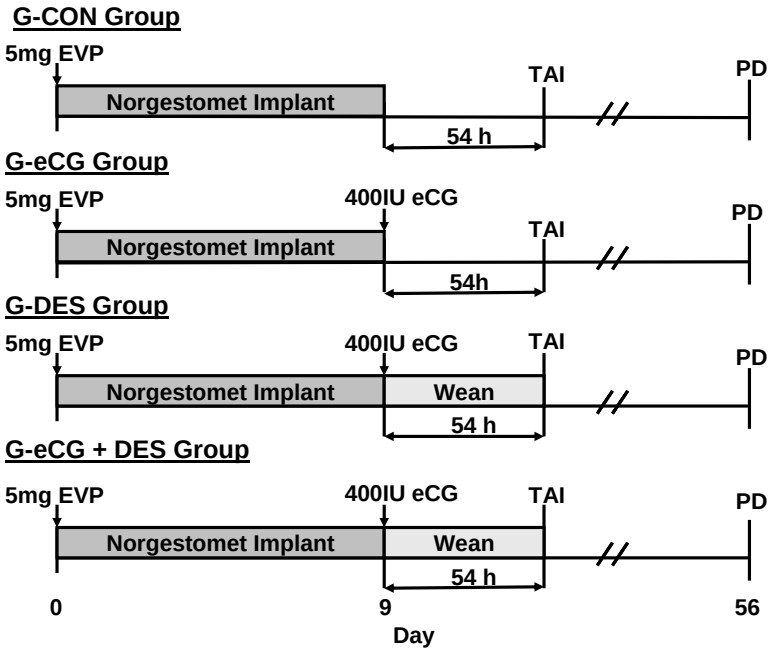


Figure 2.9 Experimental protocol as described in Penteado et al. (2004). EV = Estradiol valerate; Norgestomet implant = subcutaneous auricular implant containing 3 mg of synthetic progestogen; Wean = calves were removed at implant withdrawal until TAI 54 h later; PD = Pregnancy diagnosis was performed by rectal palpation 45 d post-TAI.

The effects of weaning for 54 h and/or eCG administration with progestogen ear implant on pregnancy rates to TAI in lactating Nellore cows were evaluated (Figure 2.9; Penteado et al., 2004). The results indicated that eCG treatment at progestin implant removal and weaning for 54 h increased pregnancy rates to TAI in lactating Nellore cows.

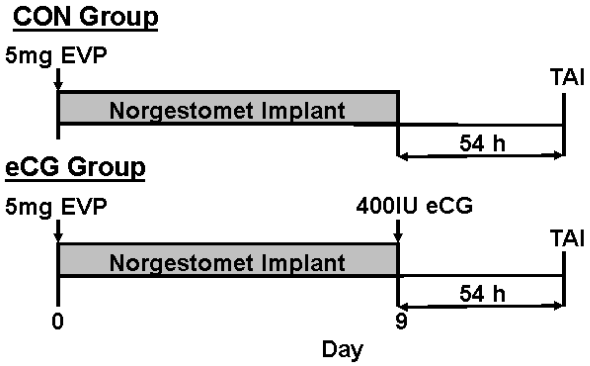


Figure 2.10 Experimental protocol as described (Rodrigues et al., 2004; Ayres et al., 2007). EV = Estradiol valerate; Norgestomet implant = subcutaneous auricular implant containing 3 mg of synthetic progestogen.

The G-CON and G-eCG progestin-based treatments used by Penteado et al. (2004) were applied to Nellore cows to determine pregnancy rates after treatments were applied to cows of varying postpartum intervals from calving to treatment (Figure 2.10; Rodrigues et al., 2004). Administration of eCG at removal of a norgestomet ear implant significantly increased pregnancy rates in cows regardless of when the treatment was applied during the postpartum period. In contrast, eCG administration at the time of ear implant withdrawal only increased pregnancy rates in the 30 to 59 d postpartum period regardless of body condition (Figure 2.10; Ayers et al., 2007).

Treatment with eCG on d 8 concurrent with norgestomet implant removal increased pregnancy rates to a TAI protocol in Nellore heifers compared with control heifers (Sá Filho et al., 2005). In contrast, another experiment involving Nellore heifers (Sá Filho et al., 2010b) reported only eCG-treated heifers that ovulated had improved pregnancy rates, whereas the authors proposed that decreased pregnancy rates detected in non-ovulating heifers may have occurred because of insufficient uterine maturation. Both experiments found pregnancies per AI were increased in heifers receiving eCG treatment at norgestomet implant withdrawal of a TAI protocol (Sá Filho et al., 2005; 2010b).

Effects of eCG treatment at the time of norgestomet ear implant removal on pregnancy rates in suckled Nellore cows were evaluated (Sá Filho et al., 2010a). In the first of 2 experiments, a tendency for an increased pregnancy rate in response to eCG treatment was reported; however, inability to report a significant difference was likely due to an insufficient number of cows allotted to each treatment (GnRH n=26; eCG n=24). Results of a second experiment found treatment with eCG at implant removal or administration on GnRH at TAI significantly improved pregnancy rates. In summary, addition of eCG to a norgestomet-based TAI protocol

seemed to improve pregnancy rates in Nellore heifers and cows. Silva et al. (2004) utilized a similar experimental protocol in lactating Nellore cows as described previously (Sá Filho et al., 2010a). Their results indicated that treatment with eCG increased pregnancy rates similar to treatment with GnRH at TAI, in agreement with those reported by Sá Filho et al. (2010a) in Nellore heifers.

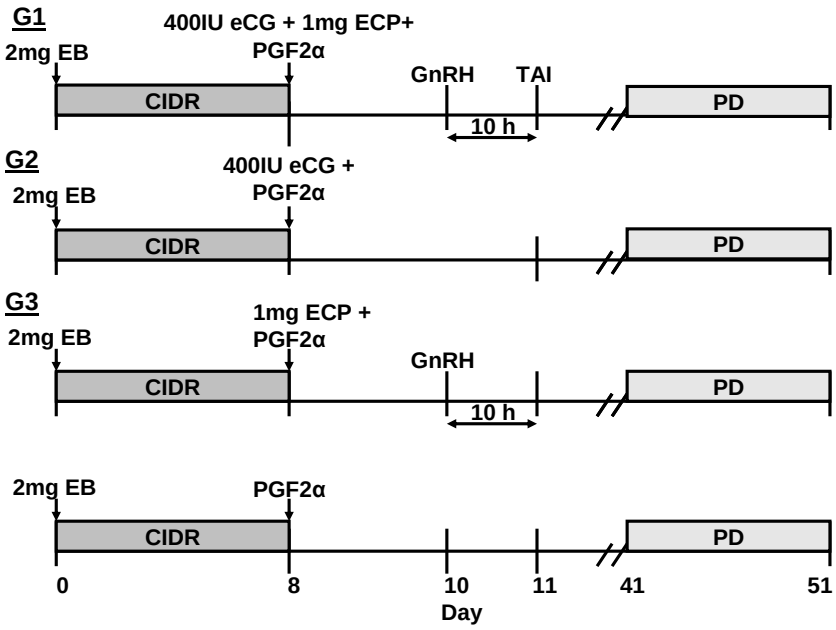


Figure 2.11 Experimental protocol as described by Souza et al. (2007b). EB = Estradiol benzoate; CIDR = Controlled internal drug release insert; ECP = Estradiol cypionate; PD = Pregnancy diagnosis was performed by ultrasonography 30 to 40 d post-TAI.

Effects of eCG and estradiol cypionate on the LH surge and conception rates in high-producing Holstein cows to which TAI protocols were applied (Figure 2.11; Souza et al., 2007b). Regardless of the ovulatory stimulus (ECP vs. GnRH), no differences were detected in the pre-ovulatory surge of LH among treatments. Group 2 had increased pregnancy rates compared with Group 4, but pregnancy rates in Groups 1, 2, and 3 did not differ. Differences in pregnancy rates

were more evident among cows with lower BCS (<2.75). Cows receiving eCG had increased pregnancy rates compared with cows not treated with eCG. In contrast, pregnancy rates did not differ in cows with BCS >2.75 with or without eCG treatment. These results indicate that eCG increased pregnancy rates in high producing Holstein cows of lesser BCS.

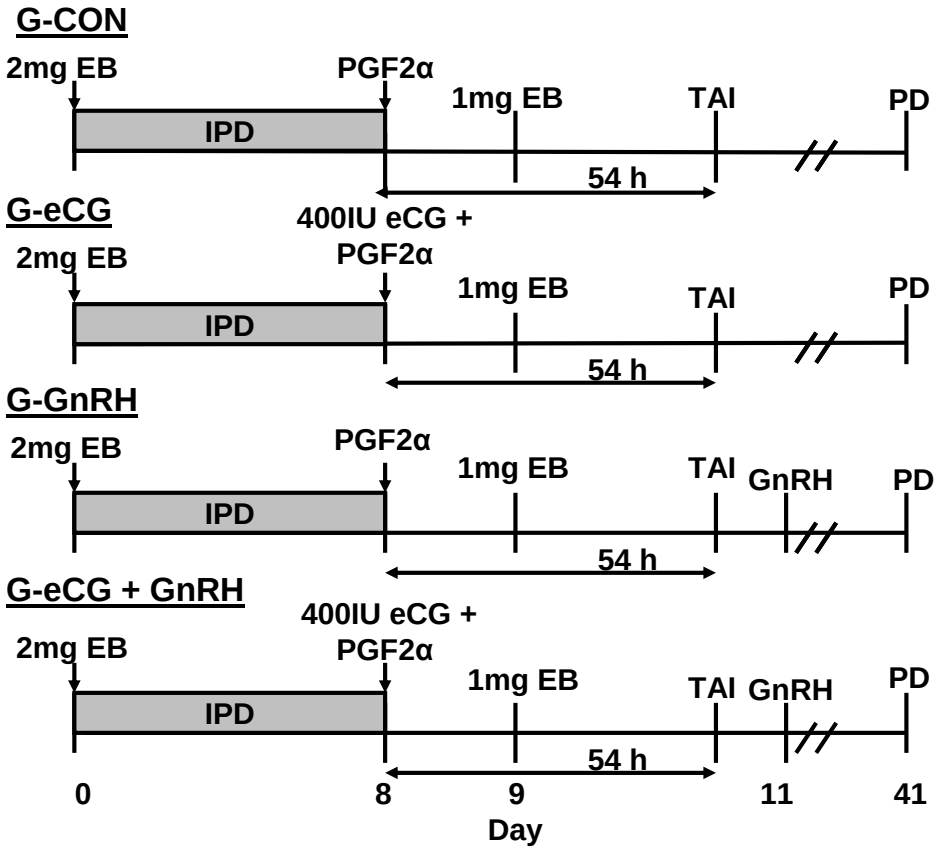


Figure 2.12 Experimental protocol as described in Duarte et al. (2004). EB = Estradiol benzoate; IPD = Intravaginal progesterone-releasing device; PD = Pregnancy diagnosis was performed 30 d post-TAI by ultrasonography.

Duarte et al. (2004) evaluated the effects of eCG administration at intravaginal progesterone insert removal and GnRH treatment to induce accessory CL 5 d post-TAI in 174 lactating Nellore cows (Figure 2.12). On d 0 all cows received EB i.m. and an intravaginal progesterone device (IPD). The G-GnRH group received 0.2 mg GnRH 5 d post-TAI. The G-eCG+GnRH

received the eCG and GnRH treatments. Pregnancy diagnosis occurred 30 d post-TAI by ultrasonography. Treatment with eCG at progesterone device removal and GnRH treatment 5 d post-TAI did not affect pregnancy rates in lactating Nellore cows.

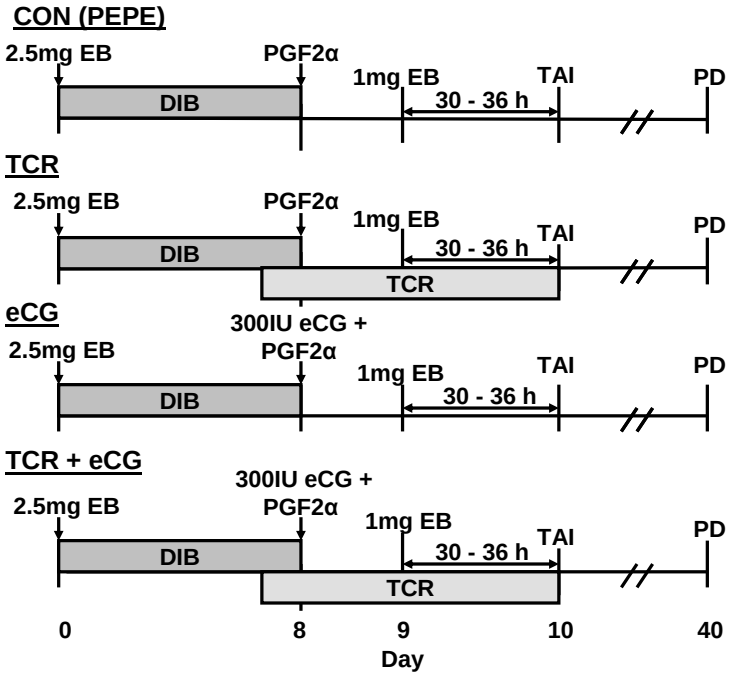


Figure 2.13 Experimental protocol adapted from Pinheiro et al. (2009). EB = Estradiol benzoate; DIB = Intravaginal progesterone-releasing device containing 1 g progesterone; TCR = Temporary calf removal occurred from device removal until TAI (54-60 h); PD = Pregnancy diagnosis determine by ultrasonography 30 d post-TAI.

Similarly, Pinheiro et al. (2006) reported temporary calf removal and/or eCG treatment did not improve pregnancy rates to a TAI protocol (Figure 2.13). Pregnancy rates were similar among all four treatments. These results indicated that treatment with eCG and/or temporary calf removal did not affect pregnancy rates in anestrous lactating Nellore cows of adequate body condition.

A subsequent study evaluated the same protocol (Pinheiro et al., 2006) in 238 cows to determine the effects of eCG and/or temporary calf removal for 54 to 60 h. Pregnancy rates were

not significantly affected by eCG treatment; however, this result may have occurred because of inadequate numbers of cows assigned to each treatment. The majority of cows used in this and the previous experiment were of adequate body condition (BCS ≥ 2.5), which may account for the difference in results obtained by others (Baruselli et al., 2003; Souza et al., 2007a). Authors failed to find an effect of eCG treatment and/or temporary calf removal on pregnancy rates in suckled Nellore cows of adequate body condition.

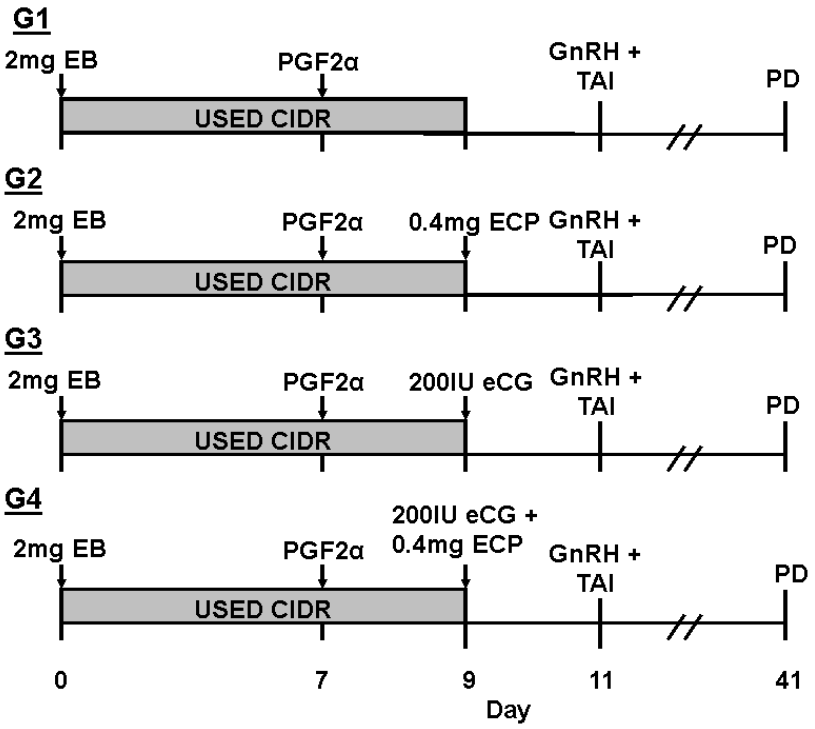


Figure 2.14 Experimental protocol as described by Vilela et al. (2008). EB = Estradiol benzoate; Used CIDR = Controlled internal drug release insert previously used for 18 d, which originally contained 1.9 g of progesterone; ECP = Estradiol cypionate; PD = Pregnancy diagnosis determined by ultrasonography 30 d post-TAI.

Effects of estradiol cypionate and/or eCG on pregnancy rates to TAI in beef heifers was evaluated by Vilela et al. (2008). Three hundred and twenty-six crossbred Nellore x Angus and 601 Nellore heifers were assigned to treatments (Figure 2.14). Pregnancy diagnosis occurred by

ultrasonography 30 d post-TAI. Treatment with eCG had no effect on pregnancy rates in crossbred heifers; however, a numerical increase was reported in Nellore heifers. Administration of 200 IU of eCG in this experiment was a smaller dose than that used earlier in heifers (Sá Filho et al., 2005; Dias et al., 2009; Sá Filho et al., 2010b) and cows (Baruselli et al., 2003; 2004; Penteado et al., 2004; Rodrigues et al., 2004; Maraña et al., 2006; Bó et al., 2007; Sá Filho et al., 2010a), which reported increased pregnancy rates after treatment with 400 IU eCG.

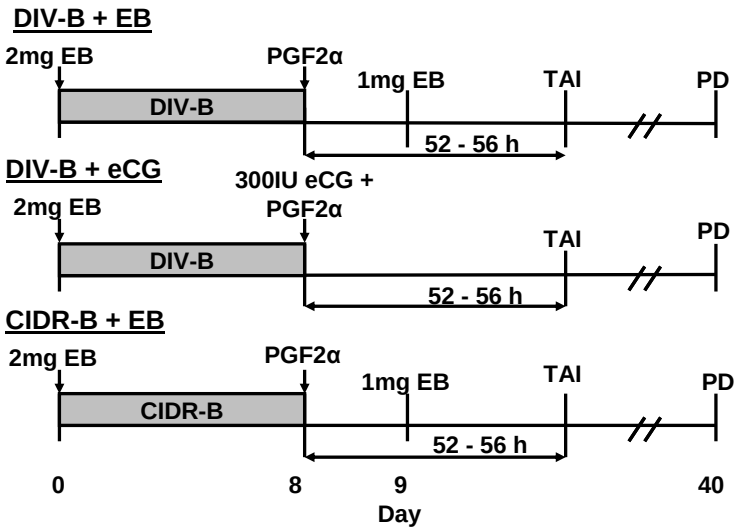
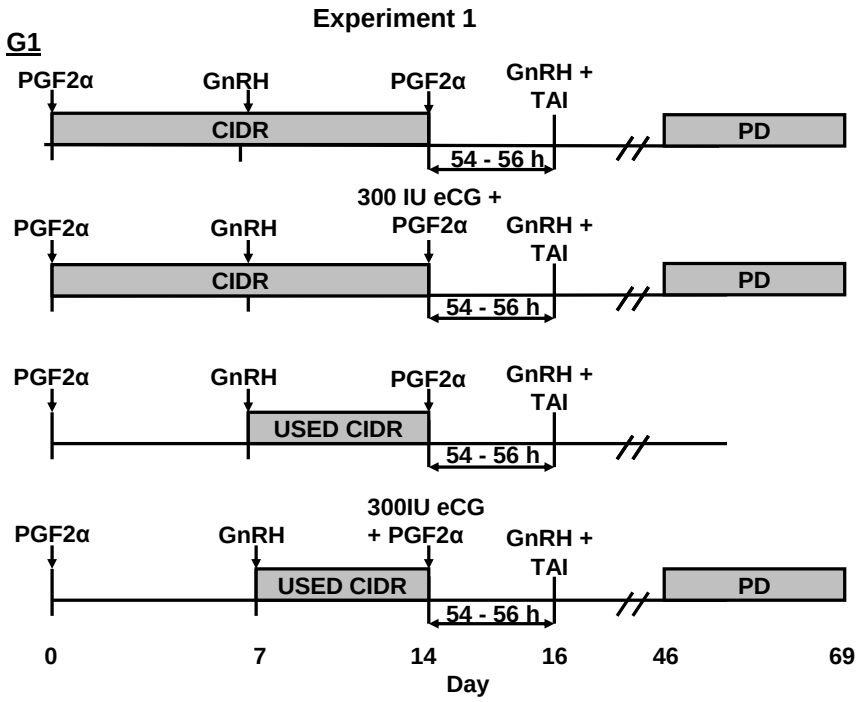


Figure 2.15 Experimental protocol as described by Bó et al. (2003). EB = Estradiol benzoate; DIV-B = Intravaginal progesterone-releasing device containing 1 g progesterone; CIDR-B = Intravaginal progesterone-releasing device containing 1.38 g progesterone; PD = Pregnancy diagnosis determined by ultrasonography 30 d post-TAI.

Bó et al. (2000) evaluated in 140 nonlactating Braford different intravaginal progesterone inserts with either eCG and/or EB on pregnancy rates in 140 nonlactating Braford (*Bos indicus*; (3/8 Brahman and 5/8 Hereford) cows (Figure 1.15). No differences were detected between inserts used. Treatment with eCG at insert removal decreased pregnancy rates compared with those treated with EB 24 h post-insert removal.

The experimental protocol of Bó et al. (2000) was re-evaluated by Cutaia et al. in 2003 using 400 IU of eCG in postpartum beef cows. Lactating Angus x Herford (*Bos taurus*) and lactating Braford (*Bos indicus*) cows were assigned randomly to 4 treatments, as previously reported (Bó et al., 2000). Treatment with eCG decreased pregnancy rates in lactating beef cows.



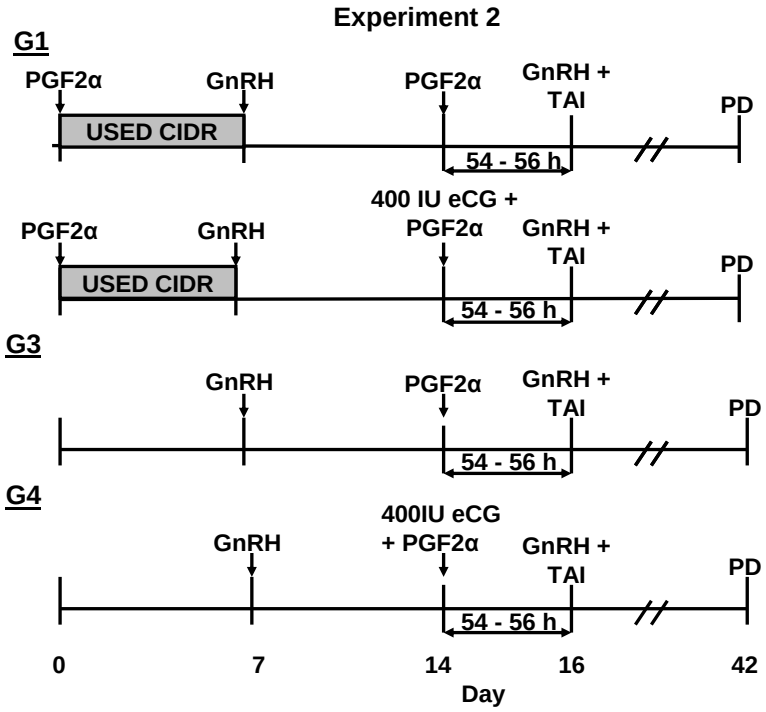


Figure 2.16 Experimental protocol as described by Colazo et al. (2006). CIDR = Controlled internal drug release insert containing 1.38 g of progesterone; Used CIDR = Controlled internal drug release device previously used and autoclaved before second use; PD = Pregnancy diagnosis was performed using ultrasonography on approximately d 50 (44 to 69 d).

The objective of Colazo et al. (2006) was to determine the effects of presynchronization and eCG on fertility to a GnRH-based TAI protocol in beef cattle (Figure 1.16). Two experiments were designed using the same experimental protocol in beef heifers (*Bos taurus*) and lactating cows. Treatment with eCG had no effect on pregnancy rates in cows and tended to reduce pregnancy rates in heifers.

The majority of results indicated that eCG treatment increased pregnancy rates in lactating beef (Baruselli et al., 2003;2004) and dairy cows (Bó et al., 2007), as well as in heifers (Sá Filho et al., 2005; Dias et al., 2009; Sá Filho et al., 2010b). Treatment with eCG at the end of a progesterone insert treatment in TAI protocols improved pregnancy rates, with greater effects in

anestrous cows (Baruselli et al., 2003; Sá Filho et al., 2010a) or cows of decreased body condition (Baruselli et al., 2004; Souza et al., 2007b). In contrast, 4 papers reported that treatment with eCG did not affect pregnancy rates in beef heifers (Vilela et al., 2008) and lactating Nellore cows (Duarte et al., 2004; Pinheiro et al., 2006; 2009); however, 3 of these reports (Pinheiro et al., 2006; Vilela et al., 2008; Pinheiro et al., 2009) administered a dose of eCG that was 100 to 200 IU less than that reported in the literature by the majority of authors. Similarly, 3 papers reported results in which eCG had no effect on pregnancy rates. Of these papers, two administered 100 IU less eCG than the more commonly administered dose of 400 IU. The majority of reported results demonstrated treatment with 400 IU of eCG as part of TAI protocols increased pregnancy rates.

Summary

Improvement in fertility in eCG treated heifers and cows may be explained by 3 primary effects: 1) eCG increased the diameter of the dominant follicle (Sá Filho et al., 2010a; 2010b); 2) eCG increased ovulation incidence (Sá Filho et al., 2004; 2005); and 3) eCG increased postovulatory circulating progesterone concentrations (Baruselli et al., 2003; Bergmaschi et al., 2005). The physiologic basis by which eCG increases fertility seems to be associated with changes in the pattern of follicular growth (Baruselli et al., 2003; Maraña et al., 2006), CL function (Marques et al., 2003; Souza et al., 2009), and CL steroidogenesis (Sá Filho et al., 2005; 2010a). Equine CG seemed to hasten follicle maturation, resulting in a superior oocyte and a more steroidogenic CL culminating in increased pregnancy rates (Baruselli et al., 2003; Bó et al., 2003).

Cows at risk of anestrus because of inadequate nutrition and poor body condition seemed to have enhanced pregnancy rates in response to treatment with eCG (Small et al., 2008; Souza et

al., 2009). Negative energy balance may affect fertility because of its association with inadequate gonadotropin secretion and inactive ovaries (Senger, 2005). Addition of eCG to TAI protocols may stimulate cyclic activity and increase the growth rate of dominant follicles in anestrous cows. The benefits of eCG seem to be more evident in more pronounced anestrous conditions (i.e., cows with only follicles <8 mm; Baruselli et al., 2003).

Because cows have preovulatory follicles of greater diameter in response to eCG treatment, Sa Filho et al. (2010) proposed that cows may have increased concentrations of estradiol; however, estradiol was not measured post-eCG treatment in any of the experiments reviewed. Elevated estradiol concentrations concurrent with decreased progesterone stimulates estrous behavior (Senger, 2005). Increased estradiol concentrations resulting from enhanced growth of the preovulatory follicle by eCG administration may lead to greater pregnancy rates. It has been reported that increased duration of estradiol secretion during follicular development before ovulation enhanced subsequent luteal lifespan (Day et al., 1990). Studies evaluating TAI protocols have reported greater conception rates in cows detected in estrus, which resulted from estrogen stimulation (Stevenson et al., 2004). We hypothesize that administering eCG would stimulate increased estradiol secretion associated with follicular maturation that would simulate the profertility effects of estradiol administration in lactating dairy cows.

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CHAPTER 3 - Ovarian Characteristics, Serum Concentrations, and Fertility in Lactating Dairy Cows in Response to Equine Chorionic Gonadotropin

Abstract

The objectives were to evaluate the effects of equine chorionic gonadotropin (eCG) administration on preovulatory follicle diameter, serum estradiol and progesterone concentrations, corpus luteum (CL) diameter, estrual activity, and pregnancy rates. Lactating dairy cows were submitted to a Presynch-Ovsynch timed artificial insemination (TAI) protocol. Cows (n = 121) in a single herd were treated with 2 injections of prostaglandin $F_{2\alpha}$ (PGF) 14 d apart (Presynch), with the second injection administered 11 d before the onset of a timed AI protocol (Ovsynch; injection of GnRH 7 d before and 56 h after $PGF_{2\alpha}$, with TAI administered 16 to 18 h after the second GnRH injection). Cows were assigned randomly to receive either saline or 400 IU eCG concurrent with the $PGF_{2\alpha}$ injection of the Ovsynch protocol (d 0). Blood samples were collected during the study to monitor serum changes in progesterone and estradiol to determine if eCG would facilitate increased estrual activity, improved ovulatory response to GnRH, and enhanced post-ovulatory luteal function. Administration of eCG tended to increase the number of CL and on d 9 and 16 after $PGF_{2\alpha}$, corresponding to d 6 and 13 post-ovulation. Volume of the post-eCG treatment luteal tissue was increased only on d 16. Timed AI pregnancy rates did not differ between eCG (36.9%) and control cows (41.8%). We concluded that use of eCG provided no fertility advantages to dairy cattle when programmed for a timed insemination at first service.

Introduction

Since 1970 the reproductive efficiency of lactating dairy cows has declined more than 50% (Butler and Smith, 1989). This decline may be explained by an inverse relationship of conception rate to milk production. Genetic selection, improved nutrition, and modern management have increased milk production per cow (Lucy, 2001). Reproductive efficiency is of great economic significance for dairy operations, because the lactation cycle is initiated and renewed by calving. Lactating dairy cows have increased steroid metabolism in response to increased milk production and DM intake, which decreases circulating progesterone and estradiol concentrations (Sangsritavong et al., 2002).

Maturing follicles have the greatest numbers of FSH receptors on d 4 of the first follicular wave of the estrous cycle (Xu et al., 1995). This is when LH receptors initially are detected in granulosa cells of the dominant follicle (Ireland and Roche, 1982). Dominant follicles respond to both FSH and LH or eCG after acquiring LH receptors (Ireland and Roche, 1982).

Administration of eCG has been shown to increase the diameter of the preovulatory follicle (Sa Filho et al., 2010b), thus suggesting an increase in circulating estradiol concentration (Sa Filho et al., 2010a). Studies evaluating AI protocols have reported greater conception rates in cows detected in estrus, which resulted from estrogen stimulation (Pancarci et al., 2002; Stevenson et al., 2004a; 2004b). Despite similar-sized pre-ovulatory follicle and corpus luteum (CL), administration of eCG increased serum progesterone concentrations in crossbred suckled beef cows (Marques et al., 2003) and dairy cows (Souza et al., 2009). It has been reported that treatment with eCG increased CL diameter and circulating progesterone concentrations 5 d after timed AI (TAI; Sa Filho et al., 2010b). By increasing the diameter of the preovulatory follicle and subsequent CL, eCG may increase blood concentrations of progesterone and estradiol, while stimulating more estrual activity.

The objective of this study was to evaluate the effects of eCG administration on preovulatory follicle diameter, circulating estradiol and progesterone concentrations, post-treatment luteal size and function, estrual activity, and pregnancy rates.

Materials and Methods

Experimental Design

All protocols involving cows used in this research were approved by the Kansas State University Institutional Animal Care and Use Committee. Lactating Holstein cows (72 multiparous and 49 primiparous) housed at the Kansas State University Dairy Teaching and Research Center were enrolled in the study. Cows were housed in covered free stalls, supplemented with overhead sprinklers and shade over the feed bunk during summer. Cows were milked thrice daily and fed twice daily a TMR calculated to meet or exceed the nutrient requirements for a lactating Holstein cow producing 50 kg of milk with 3.5% fat (NRC, 2001). The diet consisted of corn silage, sweet bran, cracked corn, alfalfa hay, whole cottonseed, soybean meal, vitamins, and minerals.

Breeding clusters were formed every 14 d as cows and heifers calved. The experiment consisted of 17 breeding clusters. Ovulation was synchronized in all cows by using the Presynch-Ovsynch protocol consisting of two $PGF_{2\alpha}$ injections (2 mL of Estrumate, Schering-Plough Animal Health, Union, NJ; or 5 mL of Lutalyse, Pfizer Animal Health, New York, NY) 14 d apart followed in 11 d by the traditional Ovsynch protocol (2 injections [GnRH-1 and GnRH-2] of 100 μg GnRH [2 mL of Fertagyl, Intervet, Millsboro, DE] 9 d apart with a 25 mg $PGF_{2\alpha}$ injection administered 56 h before a second injection of GnRH [GnRH-2]; Figure 1).

Cows were blocked by lactation number (1 to 5) and DIM, and assigned randomly to receive either 400 IU eCG (Novormon 5000, Vetrepharm Canada Inc., London, ON or Pregnecol

6000, Bioniche, Belleville, ON) concurrent with $PGF_{2\alpha}$ on study d 0 or no further treatment (control). Body condition score (1 = thin and 5 = fat; Ferguson et al., 1994) was assigned on d −7. Cows were inseminated 72 h after $PGF_{2\alpha}$ or 16 to 18 h after GnRH-2. Inseminations occurred between April and November 2009. Pregnancy was initially diagnosed 33 d post-TAI and confirmed at d 61 by transrectal ultrasonography (5.0 MHz linear-array transducer; Aloka 500 V; Corometrics Medical Systems, Inc., Wallingford, CT). A positive pregnancy outcome required presence of anechoic uterine fluid and a large CL or anechoic uterine fluid and presence of a viable embryo. Pregnancy loss was determined between the 2 pregnancy diagnoses (Figure 3.1).

Detection of Estrus

All cows were fitted with HeatWatch transmitters (Reproductive Technologies LLC, Denver, CO) on d -7 to quantify characteristics of estrus during the 96 h after $PGF_{2\alpha}$ and treatment. Transmitter function was tested on d -7 and d 0. Cows were moved from free stall pens (grooved concrete surface) into a dirt lot twice daily for 30 min during the week of TAI (d0 – 4) for visual detection of estrus. Cows were considered to be in standing estrus when 2 mounts of 2 s or longer in duration were recorded by the HeatWatch system within a 24-h period.

Ultrasonography of Ovaries

Ovaries of all cows were examined by transrectal ultrasonography to determine the structures present on each ovary on d −7, 0, 2, 4, 9, and 16 (Figure 1). A map of each ovary was drawn with the position and diameter of follicles ≥5 mm in diameter and each CL, which allowed for evaluation of luteolysis and ovulatory response to GnRH-1 on d 0, ovulatory response to GnRH-2, preovulatory follicle diameter, and CL diameters on d 9 and 16 post-treatment. Follicle diameter was determined by averaging the width and height of each follicle using the internal electronic calipers of the ultrasound machine. Ovulation on d 4 was defined as

disappearance from the ovary of 1 or more follicles greater than ≥8 mm in diameter at a site where a follicle had been recorded on the previous scan of that ovary, followed by the formation of a CL on d 9 (Figure 3.1).

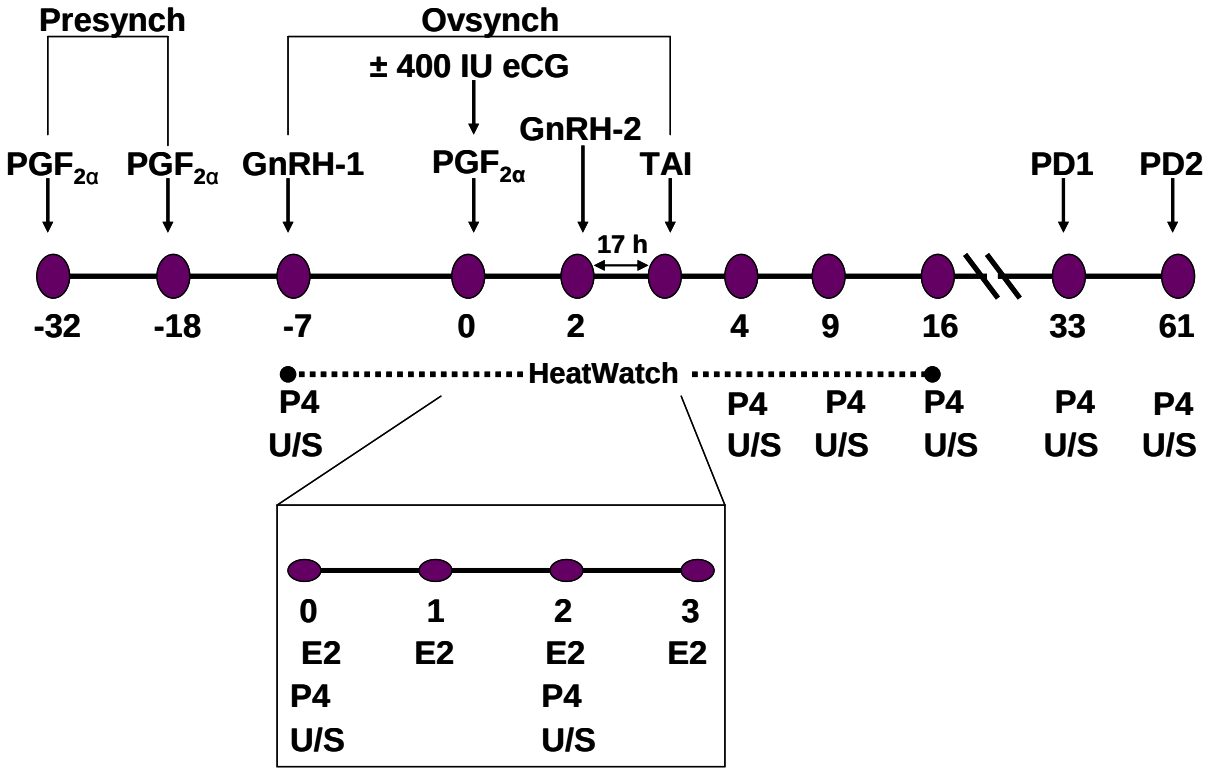

Figure 3.1 Diagram of treatments for Presynch-Ovsynch TAI protocol with or without administration of 400 IU eCG on d 0. PD = pregnancy diagnosis based on visualization of viable embryo; P4 = blood sampling and analysis of progesterone concentrations in circulating serum; E2 = blood sampling and analysis of estradiol concentrations in circulating serum; U/S = ultrasonography of the ovaries in all cows.

Blood Sampling

Blood was sampled from all cows by puncture of the coccygeal vein or artery into evacuated tubes. Blood samples were collected d –7, 0, 2, 4, 9, and 16 to assess progesterone concentrations. At pregnancy diagnosis on d 33 and 61, blood samples were collected from pregnant cows only for progesterone assay. Additional blood samples were collected every 24 h

from d 0 to 3 to assess estradiol concentrations. Samples were immediately cooled on ice and stored at 5°C for 16 h. Blood tubes were centrifuged at 1,000 x g for 15 min in a refrigerated centrifuge at 5°C to harvest serum. Serum samples were frozen and stored at −20°C until assayed for progesterone and estradiol by RIA.

Radioimmunoassays

Concentrations of progesterone in blood serum were measured in all samples by direct quantitative (nonextracted) radioimmunoassay using Coat-A-Count progesterone kits (Catalog # TKPG; Siemens Medical Solutions Diagnostics, Los Angeles, CA) and validated for bovine serum. The radioligand was ^{125}I labeled progesterone (1,500 to 2,000 μCi/μg). Specificity of the antibody (supplied by manufacturer) was nondetectable for androstenediol, estradiol, and pregnane. Specificity of the antibody was less than 3.4% for testosterone, 20α-dihydroprogesterone, 17α-hydroxyprogesterone, medroxyprogesterone, 5β-pregnan-3α-ol-20-one, 5β-pregnan-3,20-dione, 5-pregnen-3β-ol-20-one-sulfate, pregnenolone, and for 6 other common corticosteroids. Kit standards (0.05, 0.1, 0.5, 2, 10, 20, and 40 ng/mL), unknowns, and assay pools were added (100 μL each) in duplicate to antibody-coated tubes. One mL of ^{125}I labeled progesterone was added to each tube, vortexed for 5 s, and incubated overnight at 4°C. The next day, tubes were decanted, blotted on paper towels, and then radioactivity of each tube was quantified for 1 min in a gamma counter. Recovery of added mass (0.0, 0.05, 0.25, 1.0, 5.0, and 10 ng) added in quadruplicate (50 μL) to different bovine serum samples (0.066, 0.550, 3.28, and 4.70 ng/mL) averaged 102%. Parallelism was demonstrated by assaying 75- and 100-μL aliquots of bovine serum in quadruplicate and recovering 91% added mass. Samples were quantified in 6 different assays. Inter- and intra-assay coefficients of variation were 7.85 and 7.89%, respectively, and assay sensitivity was 5 pg/mL.

Concentrations of estradiol-17β were measured by radioimmunoassay (Perry et al., 1991) in blood serum with some modifications. Blood serum before assay was extracted by 10 volumes of methyl T-butyl ether (HPLC grade, Fisher Scientific, St. Louis, MO). The radioligand (^{125}I labeled 17ß-estradiol; 1,500 to 2,000 µCi/µg) was purchased from MP Biomedicals LLC (Solon, OH). Recovery of added mass (1, 2, 4, 8, and 16 pg) added in quadruplicate to 3 different bovine serum samples (300 µL) averaged 109%. Parallelism was demonstrated by assaying 150-, 200-, 250-, and 300-µL aliquots of bovine serum in quadruplicate and recovering 102% of that assayed in the 300-µL sample of bovine serum. Inter- and intra-assay coefficients of variation for assays were 5.86 and 5.82%, respectively, for a pooled serum sample that averaged 6.40 ± 0.38 pg/mL.

Statistical Analyses

Repeated measure variables, including concentrations of estradiol and progesterone, CL volume and CL number were analyzed by ANOVA (procedure MIXED, SAS Inst. Inc., Cary, NC). The model used to analyze estradiol concentration included treatment, day (study d 0, 1, 2, and 3), treatment by day, and season (April to August or September to November). The model for analyzing progesterone on study d 0, 2, and 4 was similar except for differing days. Number and volume of CL and progesterone concentration assessed on d 9 and 16 post-treatment were analyzed by using a similar model in addition to including pregnancy status assessed on d 33 (1 = pregnant or 0 = not pregnant).

Regression of the CL determined by ultrasonography (visual disappearance of a previously mapped CL) on d 2 and by changes in progesterone concentration (≥1 ng/mL at d 0, then <1 ng/mL on d 2) was assessed by logistic regression (procedure LOGISTIC, SAS Inst. Inc.). The model initially included treatment, lactation number (1 or ≥ 2), median BCS (≤ 2.25 or > 2.25), median energy-corrected milk (**ECM**, ≤46 or ≥ 46 kg/d), and season (Hot: April-August;

Cold: September-November). Presence or absence of uterine fluid on d 2 was assessed using the same model with the addition of estradiol concentration on d 2, and whether cows had low (< 1 ng/mL) or high (≥1 ng/mL) progesterone on d 2. The final models produced by backward stepwise selection of independent variables retained in each of the previous logistic regression models were based on the Wald statistic (P > 0.10) and consisted of treatment and season.

Pregnancy outcomes at d 33 and 61 and pregnancy loss were analyzed by procedure LOGISTIC (SAS Inst. Inc.) using the initial model of treatment, lactation number, their interaction, cycling status, treatment by cycling status, technician, sire, BCS, ECM, and season. The final model consisted of treatment.

Single or multiple ovulation responses to GnRH-1 and GnRH-2, preovulatory follicle diameter, and HeatWatch characteristics were assessed by using ANOVA (procedure GLM; SAS Inst. Inc.). The model included treatment, lactation number, BCS, ECM, and season.

Results

Before treatment, incidence of single (P = 0.52) and double ovulation (P = 0.86) in response to the GnRH-1 injection on d -7 did not differ in cows assigned to receive eCG or remain as controls, respectively (Table 3.1). Regression of the CL after $PGF_{2\alpha}$ injection on d 0 did not differ between treatments regardless of method employed: visual determination by observation of the CL via transrectal ultrasonography (P = 0.51) or by changes in serum progesterone concentration (P = 0.89; Table 3.1).

Table 3.1 Effect of eCG on steroid concentrations, ovarian structures, and synchronization in lactating dairy cows

	Treatment[1]		
Item	eCG	Control	*P*-value
GnRH-1[2]			
Ovulation[3], %	63.6	69.1	0.52
Double ovulation[4], %	21.4	19.0	0.86
CL regression[5],			
Visual, %	97.0	94.0	0.51
Progesterone, %	80.0	82.0	0.89
GnRH-2			
Ovulation[6], %	96.9	100.0	0.15
Double ovulation[7], %	20.3	18.2	0.73
Ovulatory follicle diameter, mm			
Primary follicle	13.9 ± 0.34	15.0 ± 0.38	0.08
Secondary follicle	11.0 ± 0.45[a]	13.9 ± 0.55[b]	0.004
CL volume, cm^3			
d 9	6.57 ± 0.51	6.97 ± 0.51	0.40
d 16	9.1 ± 0.46[a]	7.7 ± 0.51[b]	0.04
CL number			
d 9	1.3 ± 0.05	1.2 ± 0.06	0.08
d 16	1.3 ± 0.05	1.2 ± 0.06	0.09

[a-c]Values with different subscripts in the same row differ ($P < 0.05$).
[1]All cows were synchronized using Presynch followed by Ovsynch protocol with or without 400 IU eCG on d 0.
[2] Assessed 7 d prior to administration of 400 IU of eCG on d 0.
[3] Ovulation of a single follicle within 24 h in response to the GnRH-1 injection of Ovsynch.
[4] Ovulation of >1 follicle within 24 h in response to GnRH-1 injection of Ovsynch.
[5] Corpus luteum regression following PGF_{2a} administration on d 0 as determined visually (ultrasonography) and by progesterone concentration (RIA).
[6] Ovulation of a single follicle within 24 h in response to GnRH-2 injection of Ovsynch.
[7] Ovulation of >1 follicle within 24 h in response to GnRH-2 injection of Ovsynch.

Treatment with eCG did not improve incidence of single ($P = 0.15$) or double ovulation ($P = 0.73$) to the GnRH-2 injection (Table 3.1). Diameter of the largest preovulatory follicle tended ($P = 0.08$) to be greater in controls than those receiving eCG treatment. The second largest follicle diameter was greater ($P = 0.004$) in controls than in the eCG treatment (Table 3.1).

Volume of the CL on d 9 post-treatment did not differ ($P = 0.40$) between treatments, whereas CL volume on d 16 was greater ($P = 0.04$) after eCG treatment (Table 3.1). Cows treated with eCG tended to have more CL per cow on d 9 ($P = 0.08$) and d 16 ($P = 0.09$) than controls (Table 3.1).

Concentrations of progesterone decreased from d 0 to 2 after $PGF_{2\alpha}$ injection administered on d 0, but did not differ ($P > 0.41$) between treatments on d 0, 2, and 4 after eCG treatment (Figure 3.2).

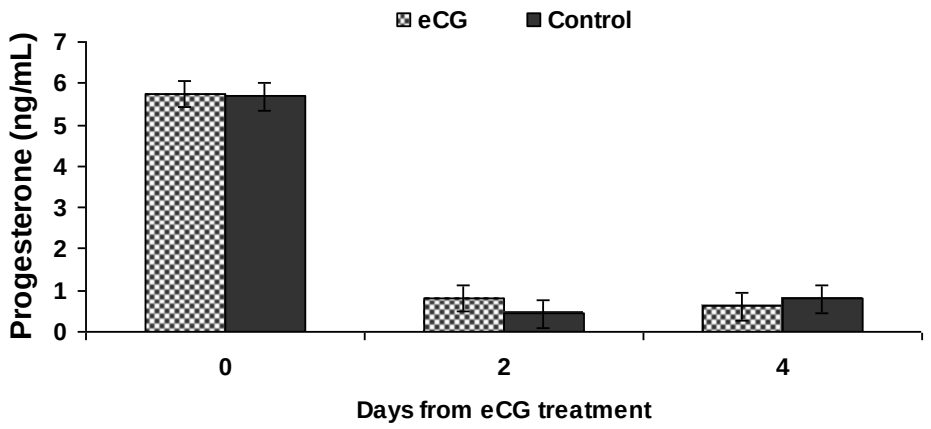

Figure 3.2 Pattern of serum progesterone concentrations on d 0, 1, 2, and 3 of the study ($P = 0.41$).

A treatment by day interaction ($P < 0.001$) was observed for estradiol concentrations during the collection period (Figure 3.3). Compared with controls concentrations of estradiol in eCG-treated cows started at a smaller concentration on d 0, but increased to a greater concentration on d 2. Thereafter, concentrations in both treatments decreased between d 2 and 3. Treatment with eCG failed ($P > 0.29$) to increase estradiol concentrations compared with controls.

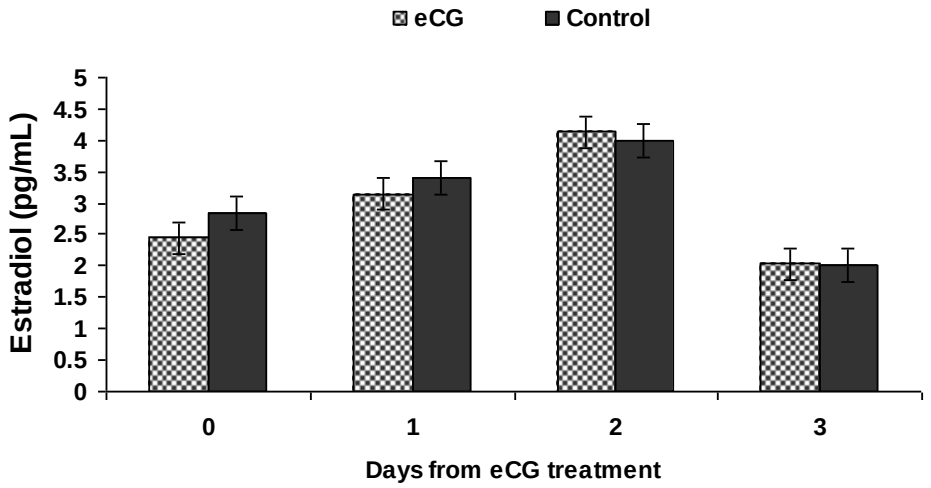

Figure 3.3 Pattern of serum estradiol concentrations on d 0, 1, 2, and 3 of the study ($P = 0.29$). A treatment x time interaction was detected on d 0, 1, 2, and 3 of the study ($P < 0.001$).

Effects of eCG treatment on estrual activity and fluid in the uterine lumen are shown in Table 3.2. Presence of uterine fluid as determined by ultrasonography on d 2 was not affected by eCG treatment ($P = 0.14$). Estrual activity was not affected by treatment and overall expression of estrus was poor (22.5%). Neither standing events ($P = 0.85$) nor average duration of standing events differed ($P = 0.85$) between treatments. Regardless of treatment, a small number of cows were anovulatory (4.2%; 5/120) before the onset of the ovulation synchronization.

Table 3.2 Effects of eCG on presence of uterine fluid and estrous activity in lactating dairy cows

	Treatment[1]		
Item	eCG	Con	*P*-value
Uterine fluid[2], %	26.3 (17/65)	18.2 (10/55)	0.14
Standing events[3], no.	3.4 ± 3.1 (10/65)	2.7 ± 3.2 (9/55)	0.85
Duration of standing events[4], s	6.7 ± 5.3 (10/65)	5.8 ± 7.4 (9/55)	0.85

[1]All cows were synchronized using Presynch followed by Ovsynch protocols with or without 400 IU eCG on d 0.
[2] Presence of fluid in the uterine lumen as determine by ultrasonography on d 2.
[3] Average number of standing events per cow recorded by HeatWatch from d 2 to 3.
[4] Average duration of standing event recorded by HeatWatch from d 2 to 3.

Serum progesterone concentrations post-AI on d 9, 16, 33, and 61 are shown in Figure 3.4. Cows receiving eCG treatment did not have increased concentrations of progesterone on d 9 (P = 0.79) and 16 (P = 0.43). Further, serum progesterone concentrations for cows diagnosed pregnant on d 33 (P = 0.81) and 61 (P = 0.42) did not differ between treatments.

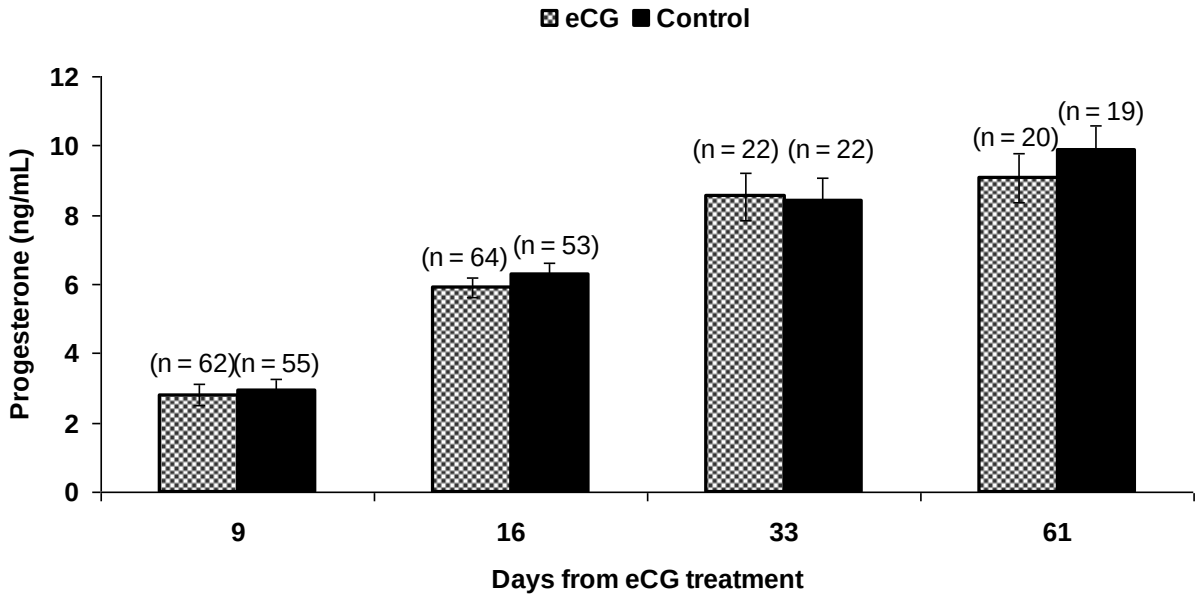

Figure 3.4 Serum progesterone concentrations post-AI on d 9, 16, 33, and 61 of the study (P > 0.42). Number of cows per bar are listed above each bar. Only pregnant cows were sampled on d 33 and 61.

Pregnancy rates were unaffected by administration of eCG determined at pregnancy diagnosis on d 33 ($P = 0.58$) or d 61 ($P = 0.96$; Table 3.3). Pregnancy loss between the first and second pregnancy diagnoses was numerically less for the eCG treated cows; however, no significant difference was detected between treatments (Table 3.3).

Table 3.3 Effect of eCG treatment on pregnancy rate and loss in lactating dairy cows

	Treatment[1]		
Item	eCG	Con	*P*-value
Pregnancy rate[2], %	(n = 64)	(n = 53)	
d 33	36.9	41.8	0.58
d 61	32.3	32.3	0.96
Pregnancy loss[3], %	4.6	9.1	0.34

[1]All cows were synchronized using Presynch followed by Ovsynch protocols with or without 400 IU eCG on d 0.
[2] Determined by transrectal ultrasonography of fluid within the uterus and the presence of a viable embryo.
[3] Determined by the absence of fluid within the uterus and the presence of a viable fetus on d 61 when pregnancy was confirmed on d 33.

Discussion

The present study provides information regarding the ineffectiveness of eCG treatment to increase the fertility of lactating dairy cows. The hypothesis that eCG treatment would increase preovulatory follicle diameter, circulating estradiol and progesterone concentrations, post-treatment luteal size on d 9, estrual activity, and pregnancy rates was not supported by results. Treatment with eCG did not increase preovulatory follicle diameter, estrual activity, circulating estradiol or progesterone concentrations, or pregnancy rates. Diameter of the CL was increased ($P = 0.04$) after eCG treatment on d 16, but not differ between treatments on d 9 of the study.

Several studies have reported that eCG either increased (Sá Filho et al., 2005) or had no effect (Small et al., 2009; Souza et al., 2009) on diameter of the preovulatory follicle. In the present study, there were no significant effects of eCG on preovulatory follicle diameter;

however, a tendency ($P = 0.08$) was detected for controls to have a larger follicle diameter than eCG-treated cows. Our results are similar to those reported in dairy cattle (Vereranada et al., 2006; Souza et al., 2009), in which eCG had no effect on the diameter of the ovulatory follicle. The second largest follicle was larger in untreated cows. Increased diameter of the second follicle may indicate controls had decreased concentrations of circulating inhibin (et al., 2001) or in response to reduced negative feedback of LH because of elevated progesterone metabolism in lactating dairy cows (Wiltbank et al., 2006).

Follicles of greater diameter secrete increased concentrations of estradiol (Perry et al., 2005). As expected, a treatment by day interaction was observed for estradiol concentrations in eCG-treated cows. Initially, estradiol concentrations started at a lesser concentration on d 0, but increased to a greater concentration on d 2. The circulating concentrations of estradiol measured from d 0 to 2 reflected a proestrous environment during which increasing amounts of estradiol are produced by follicles until a threshold level is reached. Concentrations of estradiol in both treatments decreased between d 2 and 3. The pattern of circulating estradiol reflects the decrease of estradiol secretion following the LH surge induced by the GnRH-2 injection on d 2 and subsequent ovulation.

Targets of estradiol are the reproductive tract and brain centers controlling estrual behavior. Cows exhibit increased estrual activity after treatment with eCG (Perea et al., 2008). In the present study, estrual activity was not affected by eCG treatment and general expression of estrual activity was poor (22.5%). These results are in contrast to reports of increased estrual activity after eCG treatment in beef heifers (Vilela et al., 2008). Duffy et al. (2004) reported that eCG treatment resulted in similar estrous responses to treatment with estradiol benzoate (**EB**). The current results are in agreement with Souza et al. (2009), who reported poor expression of

estrus following eCG treatment in lactating Holstein cows. Treatment with eCG did not affect estrual activity by means of the number of standing events ($P = 0.85$) or average duration of standing events.

Equine CG administration may reduce variation in ovulation timing (Cavalieri et al., 1997) and increase the incidence of ovulation resulting in improved fertility to TAI protocols (Baruselli et al., 2004; Sá Filho et al., 2005; 2010b). In addition, Duffy et al. (2004) reported increased multiple ovulations in cows receiving 600 IU eCG before the selection stage of follicle development. In the current study, treatment with eCG did not improve incidence of single or double ovulation to the GnRH-2 injection. Souza et al. (2009) reported that eCG had no effect on the percentage of cows that ovulated or the timing of ovulation, which was confirmed by the current results.

Follicles of increased diameter form CL of increased diameter and luteal activity in response to eCG treatment (Sá Filho et al., 2005). It has been reported (Souza et al., 2006) that eCG administration increased the volume of the newly formed CL. In the present experiment, volume of the CL on d 9 did not differ between treatments, whereas CL volume on d 16 was greater after eCG treatment. Rapid growth of the CL occurs between 3 and 7 d after ovulation (d 0). It is during this time period that CL volume was unaffected by eCG treatment on d 9 (7 d post-ovulation). Growth of CL luteal tissue by hypertrophy of large luteal cells and hyperplasia of small luteal cells is consistently increasing from d 7 until d 16 post-ovulation, at which time luteolysis begins. Farin et al. (1988) confirmed in ewes that hCG can induce differentiation of small luteal cells to large luteal cells. Because of the increase in CL volume on d 16 of the study, it seems that eCG may have had an effect on the population of granulosa and theca cells, which subsequently form small and large luteal cells.

Two studies (Marques et al., 2003; Bergamaschi et al., 2005) reported that eCG increased plasma progesterone concentrations without an increase in the diameter of the preovulatory follicle or CL. In the current study, administration of eCG failed to increase the circulating progesterone concentrations. These results confirm similar conclusions (Souza et al., 2009) in which eCG treatment had no effect on CL volume or circulating progesterone concentrations.

In a limited number of experiments, eCG has been given to lactating dairy cows with mixed results. One study found the addition of eCG to a TAI protocol improved pregnancy rates in cows in a pasture-based system (Bó et al., 2007). Application of a TAI protocol using EB in conjunction with eCG increased pregnancy rates in lactating dairy cows, but not in those administered GnRH and eCG (Veneranda et al., 2006). Another reported eCG treatment may increase pregnancy rates in high-producing cows with decreased body condition (Souza et al., 2007). In the present study, pregnancy rates were similar on d33 and 61 regardless of treatment. Our findings are in contrast to those reporting eCG treatment to increased pregnancy rates in lactating Nellore (*Bos indicus*) cows (Baruselli et al., 2003; 2004; Penteado et al., 2004; Silva et al., 2004).

In conclusion, efficacy of eCG varies depending on body condition (Souza et al., 2007) and cycling (Baruselli et al., 2003;2004) status in addition to other reproductive management strategies, such as administration of EB (Duffy et al., 2004) or insertion of a intravaginal progesterone releasing insert (Cutaia et al., 2003). Cows receiving eCG had CL of increased volume on d 16 but not on d 9 compared with controls, which may be caused by modulation of luteinization by eCG. Results of the present study failed to support the findings that eCG administration might improve fertility in lactating dairy cattle when programmed for a TAI at first service. This lack of benefit may be explained by adequate nutrition, low incidence of

anestrus (4.2%; 5/120), and administration of GnRH. We conclude that use of eCG provided no profertility advantages to dairy cattle when programmed for a timed insemination at first service.

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