



Equine chorionic gonadotropin: an enigmatic but essential tool

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Abstract

Equine chorionic gonadotropin (eCG) was discovered more than 80 y ago as a factor found in circulation of the pregnant mare during the first third of gestation. It is a variant of equine luteinizing hormone (LH), differentially glycosylated by the equine trophoblast cells. It has the peculiar property of provoking both follicle-stimulating hormone (FSH) and LH activity in non-equid species. The biological basis for this dual activity is believed to be the result of promiscuity of the mammalian FSH receptors, imparting the capacity to respond to this equine LH-like hormone. The best approximation of the role of eCG in the mare is that it induces accessory corpora lutea to better support early gestation. There are numerous applications for eCG in domestic species, including induction of puberty, reversal of anestrus, superovulation and, most recently, improvement of fertility.

Keywords: equine chorionic gonadotropin, follicle stimulating hormone, gonadotropin receptor, luteinizing hormone, ovary, trophoblast.

Introduction

Large animal research has been a major engine of discovery in reproductive biology. In the early days of gonadotropin research it was shown that transplantation of anterior pituitary tissue from domestic species into laboratory animals evoked precocious puberty and other reproductive consequences (Lunenfeld, 2004). The first purification, in the late 1960's, of the hypothalamic factor, gonadotropin releasing hormone (GnRH), came from extracts of hypothalami of pigs (Schally *et al.*, 1971). An earlier manifestation of this tradition was the discovery, in 1930, by Cole and Hart that serum from gestating horses injected into laboratory animals stimulated ovarian growth (Cole and Hart, 1930). The bioactive component of this serum was named pregnant mare serum gonadotropin (PMSG) to reflect the occurrence in circulation during the 2nd to 5th months of pregnancy. Somewhat later, the substance responsible for this ovarian stimulation was localized to the equine uterus, resulting in the suggestion that it was of fetal provenance (Catchpole and Lyons, 1934). Studies in the early 1970's confirmed that the source of the hormone was fetal chorionic cells that invaded the uterine epithelium to form the endometrial cups (Allen and

Moor, 1972). This discovery led to renaming the hormone with its current nomenclature, equine chorionic gonadotropin (eCG). Protein analysis demonstrated that eCG is synthesized and secreted as the classic α and β chain heterodimer, and, as with pituitary gonadotropins, it is posttranslationally glycosylated (Moore and Ward, 1980). Amino acid sequencing revealed an unexpected finding, that the primary structure of eCG β is identical to equine LH β (Sugino *et al.*, 1987). Both display a carboxyl terminal extension of 30 amino acids, and this tail is heavily glycosylated (Sugino *et al.*, 1987; Fig. 1). Later studies demonstrated that they are both the products of a single gene (Sherman *et al.*, 1992). Differential glycosylation distinguishes them, as the equine LH β chain is replete with sialylated and sulfated oligosaccharides, while the sialylated forms predominate in the more heavily glycosylated eCG β molecule (Matsui *et al.*, 1991). Differential expression of glycosylation enzymes in the chorionic girdle cells relative to the adenohypophysis is the source of the variation in the carbohydrate properties of the two hormones (Smith *et al.*, 1993). This very difference, particularly in the attachment of sialic acid, imparts to eCG one of the most important properties, its long biological half-life (Martinuk *et al.*, 1991). Indeed, equine LH is removed from circulation six fold more rapidly than eCG (Smith *et al.*, 1993).

What are the patterns of eCG secretion during equine gestation?

The chorionic girdle begins to develop in the equine embryo early in the 7th week of gestation and begins to invade the endometrium to form the endometrial cups (Allen and Moor, 1972). It is at this time, or just following, that eCG can first be detected in the serum of the mare (Catchpole and Lyons, 1934). While a general pattern exists whereby circulating levels of eCG peak at 70-80 days of pregnancy (Fig. 2), considerable variation exists between mares, in both the timing of the peak and the amount of eCG found in serum (Murphy and Martinuk, 1991). The variation in the abundance of circulating eCG has been attributed to genetic factors, with both paternal (Manning *et al.*, 1987) and maternal (Martinuk *et al.*, 1990) influences. The lifespan of the eCG producing cells appears fixed, as ectopically implanted chorionic girdle cells transplanted to non-pregnant mares persist and sustain eCG secretion for 75-100 days (de Mestre *et al.*, 2011), similar to their persistence in the uterus.

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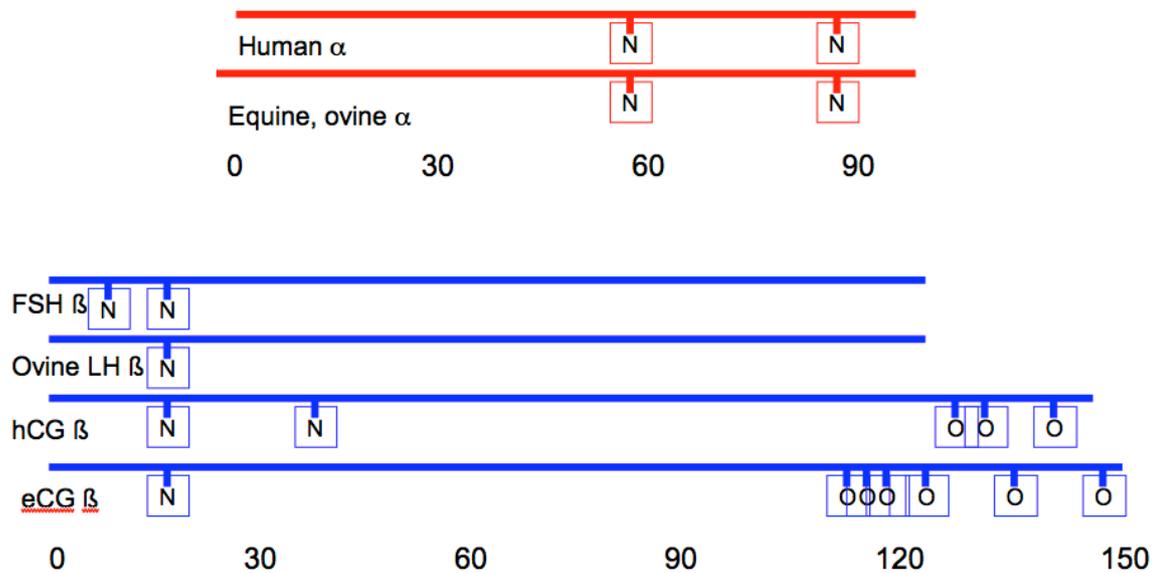


Figure 1. Linear structure of mammalian gonadotropins showing sites of attachment of carbohydrates. The latter two, hCG and eCG have C-terminal extensions that are heavily glycosylated.

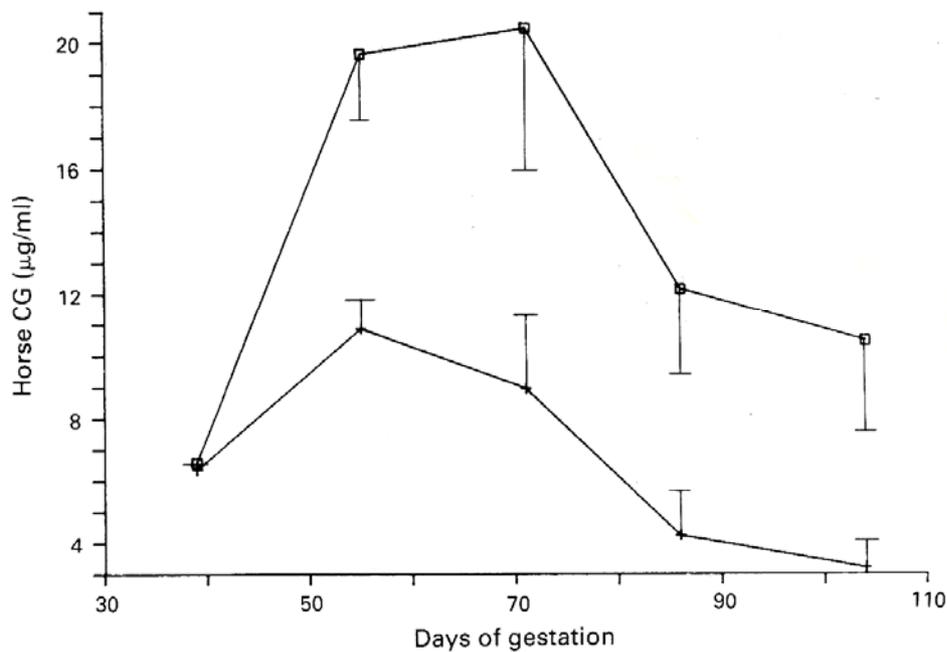


Figure 2. Mean circulating concentrations of eCG in mares sampled from days 40-110 of gestation segregated into two groups of animals, those that produce high levels of the hormone and those that have a lower secretion profile. From Manning *et al.*, 1987.

What is the role of eCG in the mare?

It is well known that the cognate embryonic glycoprotein, human chorionic gonadotropin (hCG) is the fetal signal for rescue of the corpus luteum from demise, thus maintaining gestation in the human species. Although several roles have been proposed for

eCG in the mare (Murphy and Martinuk, 1991) none has been confirmed with any degree of certainty. The equine LH receptor shows elevated homology with other mammalian LH receptors, as well as the capability to bind LH from other species (Saint-Dizier *et al.*, 2011). While eCG interacts exclusively with the equine LH receptor in the horse, it displays only a fraction of



the LH bioactivity of equine LH (Saint-Dizier *et al.*, 2004b), for reasons that are currently unclear. This notwithstanding, eCG binds to the equine corpus luteum during early gestation (Saint-Dizier *et al.*, 2004a), and thus, may provide gonadotropin support to maintain pregnancy. At approximately day 40 of gestation, accessory luteal structures appear in the ovary of the mare, coincident with, or not long after, the initiation of secretion of eCG from the endometrial cups (Amoroso *et al.*, 1948). These appear to be the result of accessory ovulations, as their initiation has been shown to be accompanied by the occurrence of oocytes in the oviduct (Amoroso *et al.*, 1948), and by concurrent increases in circulating progesterone. The temporal relationship between these two events suggests eCG may induce supplementary ovulations and support the second wave of corpora lutea (Allen, 2001), thereby ensuring that sufficient hormonal support for continuation of gestation. In bred horses hysterectomized during early pregnancy, accessory corpora lutea fail to appear (Squires *et al.*, 1974), further suggesting that eCG is the stimulus for supplementary ovulations. In hybrid gestations of donkey sire and horse dam, eCG production is remarkably diminished (Allen, 1975), with a concurrent increase in frequency of abortion during early pregnancy (Boeta and Zarco, 2010).

A second purported function of eCG in the mare has been stated to be isolation of the semi-allogenic fetus from recognition by the maternal immune system. Mammalian trophoblast in general has this capacity, first by suppression of the maternal immune system and then by alteration of maternal immune cells to participate in, rather than antagonize, implantation. Equine trophoblast, and, in particular, the chorionic girdle cells, have this capability, as demonstrated by persistence following allogenic transplantation to non-pregnant mares (de Mestre *et al.*, 2011). There is no evidence that eCG participates in this immuno-isolation, indeed, paternal antigens are recognized by the maternal immune system as early as day 45 of gestation (Baker *et al.*, 2000).

What is the basis of the biological activity of eCG?

As noted above, eCG can function as luteinizing hormone in the horse, albeit less robustly than eLH (Saint-Dizier *et al.*, 2004b). The remarkable property of eCG that has been exploited in multiple experimental and commercial contexts, is its capacity to express follicle-stimulating hormone (FSH) activity in non-equid species. While the biological basis for this phenomenon remains incompletely understood, the intuitive explanation is that the dual activity must be based on structural determinant of eCG or of the LH and FSH receptors in non-equid species.

One of the characteristics of eCG is the extended length of its effects. When injected into rats,

eCG displays an elimination half-life in excess of 5 h (Aggarwal and Papkoff, 1985), while, in sheep, this value is 21 h (Martinuk *et al.*, 1991; Fig. 3). In cattle, the half-life of disappearance was estimated to be 45.6 h (Schams *et al.*, 1978). The basis for this persistence in circulation is the extensive glycosylation of the molecule, comprising some 45% of its molecular weight (Papkoff, 1974; Matteri *et al.*, 1987). Both chains are glycosylated at asparagine-linked (N-glycosylation) and serine-threonine (O-glycosylation) sites and the C-terminal extension of the β -chain is heavily glycosylated (Smith *et al.*, 1993). It is the N-glycosylation, in which the carbohydrate chains contain sialic acid, that confers the long half-life (Martinuk *et al.*, 1991). Sulfated (O-linked) glycans are recognized by the liver and rapidly cleared, while sialylated glycoproteins persist (Green *et al.*, 1986). Removal of sialic acid from eCG by neuraminidase treatment reduces clearance time (Fig. 3) and removal of 80% reduced this time to less than 60 min (Martinuk *et al.*, 1991). This notwithstanding, the pattern of glycosylation of eCG dictates not only its persistence in serum but also its biological activity in the target tissue (Martinuk *et al.*, 1991; Murphy and Martinuk, 1991; Ulloa-Aguirre *et al.*, 1999).

The α -chain of the glycoprotein hormones is not exclusive to FSH or LH, rather, it is also present in thyroid stimulating-hormone, hCG and eCG. While both the α and β chains of eCG are necessary for biological activity (Papkoff, 1974), the β -chain confers ligand specificity to these hormones. The tertiary structures of mammalian FSH and LH, determined by x-ray diffraction, indicate that they share the same overall folding patterns, in spite of considerable difference in primary structure of the β -chains. Replacement of the α -eCG with α -chains of other mammalian species inactivates eCG, but, surprisingly a hybrid of α -eCG and porcine β -LH is many fold more potent than the native porcine LH (Bousfield *et al.*, 1985). While the capacity of eCG to induce both FSH and LH activity is therefore expected to be due to some characteristic of its β -chain, primary, secondary or tertiary structure it may also be related to the structure of the α - β heterodimer. Alternatively, posttranscriptional glycosylation may play a role in the dual bioactivity. The secondary structure of eCG, as with other gonadotropins, is influenced by 10 half-cystine residues that can form disulfide bonds (Hearn and Gomme, 2000). Three of these disulfide bonds form the so-called cystine knot on the gonadotropin β -chain (Alvarez *et al.*, 2009). A further β -chain disulfide interaction results in formation of a loop known as the seatbelt, that interacts with the α -chain (Hearn and Gomme, 2000), and contains a 10 amino acid sequence, given the name the determinate loop. These regions are believed to be essential to biological specificity of gonadotropins (Moyle *et al.*, 1994). A bifunctional gonadotropin, i.e. with both FSH

and LH activity, can be formed by introducing sequences from both gonadotropins into the determinant loop region (Moyle *et al.*, 1994). Indeed, by mutation of

amino acids within a disulfide loop in the seatbelt region, the FSH activity of eCG was decreased, without compromise of the LH activity (Galet *et al.*, 2009).

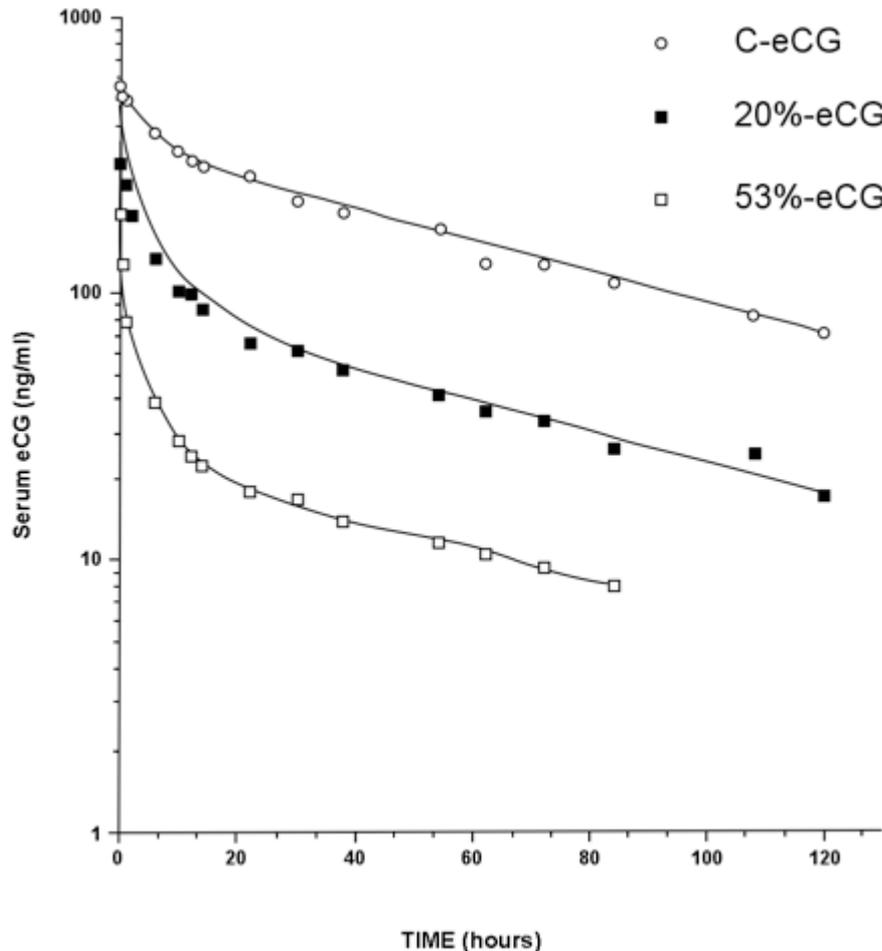


Figure 3. Disappearance rates of a bolus dose of eCG treated with neuraminidase to reduce sialic acid and infused into ewes. C-eCG represents the control, intact eCG, and 20 and 53% -eCG represent differential reductions in the carbohydrate content. Redrawn from Martinuk *et al.* (1991) with permission.

Specificity of gonadotropin receptors is conferred by two contact sites on the large extracellular domains that interact with unique regions of the cognate ligand (Moyle *et al.*, 2004). These regions are believed essential for transduction of the gonadotropin signal (Moyle *et al.*, 2005). Minor modifications by site-directed mutagenesis of the FSH receptor result in an ability to bind hCG with high affinity (Lin *et al.*, 2007). Further experimentation has revealed that the FSH receptor contains a cryptic binding site capable of binding hCG (Lin *et al.*, 2007).

Naturally occurring mutations in the human FSH receptor render it capable of binding hCG, the human placental LH (Vassart and Costagliola, 2011). Surprisingly, these mutations are not found in the extracellular domain, rather in the transmembrane loops, the region that conveys the signal to the interior of the cell (Montanelli *et al.*, 2004). The mechanism by which

intrahelical mutations induce receptor promiscuity is believed to be related to their capacity to increase the constitutive activity of the receptor (Vassart and Costagliola, 2011), thereby allowing LH-like hormones to interact with cryptic sites and to signal through the FSH receptor.

Thus, promiscuity of receptor binding, particularly in the FSH receptor, is the basis for dual activity of eCG, as its primary structure allows it to interact with this receptor. This effect is reinforced by its long half-life.

What are the practical uses of eCG?

Perhaps the most widespread use of eCG has been exploitation of its FSH activity in induction of estrus in immature animals. As the age at which pigs attain puberty can be variable, it has become standard



industry practice to advance puberty in pigs with a combination of relatively low doses of eCG (400-500 IU) to induce follicle development and 100-200 IU of hCG to induce ovulation. Treatment of immature animals in this fashion results in expression of estrus in 90% of the gilts within 6 days (Bartlett *et al.*, 2009). Although practices vary, animals are usually not bred at the induced estrus, but rather, some 21 days later at the second estrus (Martinat-Botte *et al.*, 2011). This results in marginally larger litter sizes (Bartlett *et al.*, 2009). Treatment with eCG has been less frequently employed to induce puberty in ruminants, although it has been successfully used in sheep (Sawalha *et al.*, 2011).

One of the major issues in bovine reproduction is the duration of postpartum anestrus, as its length has extensive consequences on production. The principal cause of the long intervals that precede the first estrus following parturition is reduced gonadotropin secretion, caused by suckling by the calf or undernutrition, or, more often, a combination of both. Several approaches have been taken to abbreviate anestrus, including progesterone therapy, direct stimulation of the pituitary by GnRH and treatment with eCG. Often these treatments are administered in combination (Bo *et al.*, 2003).

In the early days of embryo transfer, eCG was employed to induce superovulation in donor animals. Its long half-life confers the advantage of superovulation by means of a single injection, but the same trait also limits its usefulness, as it has the tendency to overstimulate the ovary, resulting in multiple, unovulated follicles and a yield of viable embryos that is highly variable (Bever *et al.*, 1989). Our dose response studies showed that higher doses (3600 IU/cow), while resulting in a higher number of ovulations, produced fewer transferable embryos (Gonzalez *et al.*, 1994). These studies indicated that the most efficacious dose, among those employed, was 2500 IU/cow. The reduced embryo quality has also been attributed to the LH activity of eCG, as it may cause precocious resumption of meiosis in the oocyte, resulting in aneuploidy and other problems of early embryo development (Monniaux *et al.*, 1984). A strategy that has been used to address these problems is the administration of eCG antiserum at a time point after eCG treatment (Gonzalez *et al.*, 1994). Treatment with antiserum at 60 h after eCG injection doubled both the number of ovulations and the number of transferable embryos (Gonzalez *et al.*, 1994). While this procedure entails two treatment intervals during the superovulation protocol, overall, this is much less than required by daily injection of pituitary gonadotropins. Recent variations on the superovulatory theme have included replacement of pituitary FSH with low doses of eCG (200 IU) on the two final days of the superstimulation protocol (Mattos *et al.*, 2011). This approach has proved beneficial in increasing the number of transferable embryos.

The related use of eCG has been in synchronization protocols that have allowed fixed time

insemination in cattle (Sá Filho *et al.*, 2010) and sheep (Martemucci and D'Alessandro, 2011) and in synchronization of recipient cows for fixed time embryo transfer (Bo *et al.*, 2011). For fixed time insemination, the treatment based on a low dose of eCG (300 IU) following prostaglandin or progesterone/estrogen synchronization has been applied to Zebu (*Bos indicus*) cattle, with variable results (Dias *et al.*, 2009; Butler *et al.*, 2011). Use of eCG proved advantageous in increasing pregnancy rate for fixed time embryo transfer, independent of the protocol employed for synchronization (Bo *et al.*, 2011).

How does eCG at low doses improve reproductive outcomes?

As described above, eCG displays both FSH and LH activity, and it is known that both of these hormones are required for the peri-ovulatory maturation of the mammalian follicle. A number of studies have shown that this treatment increases the frequency of multiple ovulations and results in elevated circulating progesterone, but, in some studies, increased progesterone does not correlate with increased pregnancy rate (Nogueira *et al.*, 2004). However, in some protocols, eCG-induced upregulation of progesterone synthesis has resulted in improved gestational success (Baruselli *et al.*, 2010). Currently, the only known mechanism for improved fertility with low dose treatment of eCG is this upregulation of luteal function. In a sense, this effect mimics the proposed effect of eCG in the mare, i.e. support of the accessory corpora lutea resulting in increased ovarian support of the uterus of early gestation (Allen, 2001).

A recent study comparing global gene expression in corpora lutea collected during the early luteal phase from cattle treated with 400 IU eCG just prior to termination of a fixed time protocol showed that the transcripts of more than 400 genes were in differential abundance compared to controls (Fatima *et al.*, 2011). Further analysis of this phenomenon can be found in another manuscript included in these proceedings.

Conclusions

Although we have learned a great deal about the biology of eCG since its discovery some 80 y ago, many aspects remain unknown or poorly understood. For example, it is not entirely certain what the role of eCG might be in the species of origin, the horse. Advances have been made in understanding the dual biological activity of eCG in non-equid species, but further research is required to confirm and extend current findings. These persistent enigmas do not detract from the utility of eCG in inducing folliculogenesis, promoting puberty, reversing anestrus and inducing superovulation for embryo transfer in a number of species.



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