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Review article

Natural and recombinant equine chorionic gonadotropins past and future in animal reproductive technology

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Abstract

Equine Chorionic Gonadotropin (eCG) previously named Pregnant Mare Serum Gonadotropin (PMSG) has been widely used since the 40s in animal reproduction control. It is extracted from the blood of pregnant mares between days 40 and 120 of gestation. Animal welfare organizations have voiced concerns against mares bleeding conditions. There is currently no effective substitute for the natural PMSG. In this review, we summarize the basic knowledge of the structure and biology of eCG, and the research on recombinant eCG production in the past five years.

Keywords: Chorionic gonadotropin, Equine CG, Fertility control, Glycoprotein hormone, Gonadotropins

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INTRODUCTION

Improvement in fertility control is important for both humans (infertility treatment and contraception) and farm animal species (improved reproductive efficiency). The gonadotropins stimulate gonads (ovaries or testes) gametogenesis and endocrine activities. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are secreted by the pituitary gland in all vertebrates. However, chorionic gonadotropin (CG) is only secreted by the placenta of horses and primates. Exogenous gonadotropins are widely used in the treatment of infertility in humans and animals (Lunenfeld et al., 2019), and endogenous gonadotropins are targets for contraception (Talwar et al., 2015). Gonadotropins are important in the control of the efficacy of reproduction (fertility) in domestic animals (Sousa et al., 2016). These molecules are thus important pharmaceuticals for the management of reproduction in humans and animals.

Equine CG (eCG) belongs to the glycoprotein hormones group, and has been discovered 95 years ago. eCG is secreted from syncytiotrophoblast cells of endometrial cups in pregnant mares in the first period of gestation (approximately from day 36 to day 100) (Murphy and Martinuk, 1991). Firstly, the equine trophoblast cells, which are at the chorionic girdle of the embryo, connect and invade the uterine epithelium around day 36 of gestation to shape endometrial cups (Moor et al., 1975). Next, these endometrial cups have complete structures around days 50 to 60 of gestation. Lastly, around days 70 to 80 of pregnancy, these forms degenerate (Murphy and Martinuk, 1991).

Recombinant gonadotropins generated in mammalian cells are the only ones known to exhibit biological activity *in vivo*, such as hFSH expressed in ovarian cells (Keene et al., 1989) and have been marketed as Gonal-F (Serono, Geneva, Switzerland) and Puregon (NV Organon, Oss, Netherlands). Today, the technology for the production of recombinant gonadotropins has been extended to non-mammalian systems. A major advantage of these systems is the production of recombinant proteins in larger quantities, at a lower cost and without fetal sera, such as the baculovirus system (Huang et al., 1991; Kato et al., 1998), the methylotrophic yeast system Pichia pastoris (Fidler et al., 2003), and plant systems (Dirnberger et al., 2001). However, one limitation is that the recombinant gonadotropins generated by this technology only show biological activity in vitro, no information regarding their *in vivo* potency having been reported. Later, Legardinier et al. (2005) generated recombinant eLH/CG from two insect cell lines expressing several mammalian glycosyltransferase genes, Sf9 and Mimic cells. The recombinant glycoprotein hormone eLH/CG was derived from two cell lines with LH and FSH biological activities in vitro, with similar potency to that of eCG. In contrast, they did not exhibit significant *in vivo* bioactivity.

STRUCTURE of eCG

The structure of eCG is made of an α-subunit with 96 amino acids, which bear two N-linked glycosylation sites at Asn^{56} and Asn^{82}, and a β-subunit with 149 amino acids, which possess only one glycosylation site at Asn^{13} (Figure 1). In addition to N glycans, the β-subunit also has a carboxy-terminal

peptide (CTP) of 28 amino acids, so the length of the β-subunit is extended from 121 to 149 amino acids (Figure 1). There are 10-12 O glycosylated sites on the CTP extension of the eCG β-subunit (Matsui et al., 1991; Matsui et al., 1994).

Polysaccharide chains have a vital role in the biological activity of gonadotropins because they are involved in the structure of their subunits, their excretion, and their half-life (Matsui et al., 1994; Legardinier et al., 2005). However, O-linked sites do not directly play a role in connecting the hormone to its receptor, only N-saccharides are essential for the transduction of the eCG signal. The carbohydrate content in eCG accounts for the highest percentage (more than 40%) among the family of glycoprotein hormones, which gives it an exceptional half-life compared with other hormones.
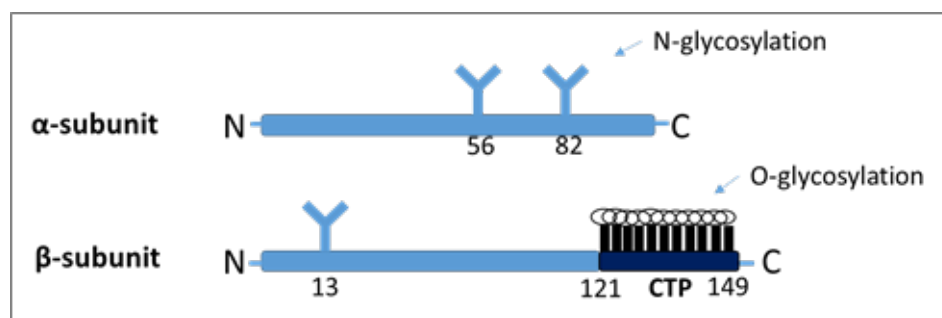

Figure 1 Types of glycosylation in the structure of the eCG hormone.

BIOLOGICAL ACTIVITIES AND FUNCTIONS OF eCG

The particular feature of eCG is that it exhibits both FSH and LH activities in non-equine species (Combarnous et al., 1978), which makes it a very interesting model to study the structure-function relationships of gonadotropins and their receptors (Combarnous et al., 1978; Combarnous et al., 1981; Guillou and Combarnous, 1983). The biological activity of eCG is controlled by the distinct structure of the N-saccharide and O-saccharide chains attached to the subunits (Matsui et al., 1994; Legardinier et al., 2005). Thus, although eCG and eLH have the same protein structures, eCG biological activity is stronger than that of eLH due to its higher molecular weight and consequently longer half-life. Carbohydrates make up 40% of the weight of eCG, while that number is only 30% in eLH (Legardinier et al., 2005). One of the characteristics of eCG is a long half-life: 5 hours in rats, 21 hours in sheep, and 45.6 hours in cattle (Murphy and Martinuk, 1991). The most important role of eCG in mares is to bind with the LH receptors in corpora lutea to maintain the pregnancy over the first period of gestation (Saint-Dizier et al., 2004). Around week 5 of gestation, accessory corpora lutea appear in mare ovaries. At this time, eCG is secreted by placental endometrial cups to stimulate these corpora lutea. eCG is then secreted from the placental endometrial cups to stimulate corpora lutea.

In non-equine species, eCG induces the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Combarnous et al., 1978). The uses of eCG include reversal of anesthesia, stimulation of puberty,

enhancement of fertility and ovulation (Murphy and Martinuk, 1991). This hormone is widely used, together with human chorionic gonadotropin, to stimulate follicular development and ovulation in immature gilts (Esbenshade et al., 1990). It is also used to induce ovulation in dairy cows, goats, sheep and pigs (Murphy and Martinuk, 1991). eCG is commonly used in artificial insemination procedures in dairy cows, where it is believed to have beneficial effects on the developing embryo (De Rensis and Lopez-Gatius, 2014). eCG is often used in combination with progesterone to induce ovulation in livestock prior to artificial insemination. eCG tends to be widely used because of its long half-life. In addition, it stimulates the maturation of the seminiferous tubules and spermatogenesis in males (Combarnous et al., 1978; Combarnous et al., 1981). During embryo transfer, eCG is used to stimulate the ovaries in donor animals. Its long half-life offers the advantage of single-injection supercharge, as it tends to stimulate the ovaries, resulting in multiple follicles and very high yields of viable embryos (Bevers et al., 1989). As described above, eCG displays both FSH and LH activities, and it is known that both of these hormones are required for follicular maturation and ovulation in mammals. Some studies have shown that this treatment increases the frequency of ovulation and leads to elevated circulating progesterone. But in some other studies, increased progesterone was not correlated with increased pregnancy rates (Nogueira et al., 2004). However, in some protocols, the eCG-induced increase in progesterone synthesis has resulted in improved pregnancy success. Currently, the only known mechanism improving fertility with low-dose eCG treatment is the enhancement of corpus luteum function (Baruselli et al., 2010).

RECOMBINANT EQUINE CHORIONIC GONADOTROPIN (rec-eCG)

For sanitary and ethical reasons, it has become more and more difficult to use natural gonadotropins for infertility treatments in humans or reproductive control in animals. It is thus mandatory to master the production and use of recombinant gonadotropins for these uses. In farm animals (cattle, pigs, sheep, goats, etc.), hormones with FSH activity (porcine FSH or eCG/PMSG) are used to increase the number of ovulated oocytes (superovulation) and thereby the number of embryos that can be collected. The availability of long-acting eCG or FSH preparations is thus important to efficiently promote folliculogenesis.

The α-subunit of eCG has 2 N-linked, while the β-subunit only includes 1 N-linked and a carboxyl-terminal peptide (CTP) containing 12 O-glycosyl potential sites among which 10 to 12 are effectively glycosylated (Matsui et al., 1991). In order to change the biological activity of rec-eCG, two approaches may be used individually or simultaneously: 1) Modifying one or more amino acids which bind to carbohydrate chains, or modifying CTP directly; 2) Using CTP as a linker to create a single-chain eCG or to connect with other gonadotropin molecules like FSH (Garcia-Campayo and Boime, 2001).

The single-chain eCG β-α (sc-eCG) was designed by using CTP on the β-subunit as a linker to the α-subunit. Therefore, there was no change in the natural eCG amino acid sequence except for the new link between the β CTP C-terminus and the α N terminus. This molecule was thus wild-type single chain eCG (sc eCG WT).

This sc eCG WT retained most biological activities of PMSG. For example, sc eCG has the complete properties of LH and FSH in non-equine species, and only those of LH in mare in in vitro bioassay (Park et al., 2009). In PathHunter Parental cell line, the binding ability of sc eCG to receptors was preserved (Lee et al., 2017), and its LH-like activity was similar to that of PMSG (Min et al., 2004; Byambaragchaa et al., 2018). The long half-life and ovarian stimulation of sc eCG also has the same PMSG (Min et al., 2004; Park et al., 2009; Lee et al., 2017; Byambaragchaa et al., 2018). However, replacing amino acid Asn56 and removing oligosaccharide residues of CTP results in a lower ovulation percentage, although there are 18% more functional oocytes in sc eCG treated mice group compared to the PMSG-treated mice group (Min et al., 2019).

Natural eCG and eLH share identical protein structures because the α and β genes which encode the α and β polypeptide chains of eCG are the same as those of eLH, although they differ widely in their glycan structures. This difference is due to their respective producing cells: pituitary gonadotrophs for eLH, and placental syncytiotrophoblast for eCG/PMSG. Consequently, the recombinant hormones, produced by eukaryote cells such as CHO, HEK, or Sf9, are identical to the natural hormones in terms of protein structure but significantly differ from both eLH and eCG for their glycanic structures (Legardinier et al., 2005).

In order to test the role of oligosaccharide chains in eCG, N-linked oligosaccharides were removed at Asn^{56} and Asn^{82} of the α-subunit, and Asn^{13} of the β-subunit. When Asn^{56} was replaced, the LH-like activity of the mutant eCG decreased significantly (Min et al., 1996). The same result was obtained when the N-linked saccharide of sc eCG was removed at Asn^{56} (Min et al., 2020; Lee et al., 2021). Therefore, the N-linked oligosaccharide at Asn56 plays a vital role in the LH-like activity of eCG. When the CTP of the β-subunit was deleted in the single-chain βxα WT, the LH-like activity of eCG was unchanged (Min et al., 1996). In another study, O-linked saccharides at the CTP of the β-subunit of the sc eCG WT were deleted. This research showed that the LH-like activity of the mutant was only slightly reduced and that the O-linked saccharides at the CTP in the β-subunit did not affect the LH activity of eCG (Min et al., 2004). Moreover, Min et al. (1996, 2004, 2020, 2021) have also shown that rec-eCG causes an increase in testosterone in leydig cells similar to that caused by eCG WT, but the estradiol levels in granulosa cells stimulated by rec-eCG were slightly lower than those stimulated by eCG WT. This means that the LH-like activity of this rec-eCG is more potent than the FSH-like activity.

In another assay, poly-histidine was tagged at C- or N-terminus at the α and β-subunits of eCG WT to create the heterodimeric mutants His α x β, α-His x β, α x β-His, and α x His-β. His-α×β and α×β-His had full in vitro LH activity, while the LH activity of α-His×β and α×His-β was reduced by 30 to 50% when compared with eCG WT (Legardinier et al., 2008). To determine the intracellular signal transduction to cAMP via LH receptors, the amino acid residues 102 - 104 of the β-subunit of sc eCG were replaced. The Rmax value of mutant eCG at amino acid 104 was the lowest, which means that this amino acid plays an essential role in the intracellular signal transduction to cAMP through LH receptors (Byambaragchaa et al., 2021b).

When amino acids 94 - 96 of the β-subunit were changed, it led to the disruption of the FSH-like activity of sc eCG (Park et al., 2010), in agreement with the negative specificity mode developed by one of us (Combarnous et al., 1981; Combarnous, 1992). These studies reveal that amino acids 102 - 104 and 94 - 96 of the β-subunit play an important role in the signal transduction to FSH receptors. In order to test the intracellular signal transduction of T-eCG to cAMP via FSH receptor, a study replacing the amino acids 102 - 104 was carried out. The results showed that the EC50 values of the eCG mutants which were measured in cells expressing FSH receptor were only 2.5 - 20% compared with those of the eCG WT (Galet et al., 2009). The same results were obtained when amino acids 104 - 109 of the β-subunit were changed. The proportions of the FSH- and LH-like activities were approximately 25% and 100%, respectively, compared to those of the sc eCG WT (Byambaragchaa et al., 2021a). These outcomes suggest that the amino acids 102 - 104 and 104 - 109 of the β-subunit indirectly affect the FSH-like activity of eCG more than the LH-like activity.

The sc eCG mutants at amino acid Asn^{56} replaced by Gln and deleted O-linked polysaccharides at the CTP of the β-subunit resulted in a rapid decrease, or even the absence, of the signal transduction to LH- and FSH receptors (Park et al., 2017; Byambaragchaa et al., 2021a). Therefore, the N and O-linked sites at Asn56 and the CTP are essential for the signal transduction to receptors. To check the biological function of the COOH-terminal amino acids in the α-subunit associated with the signal transduction, some amino acids such as Lys^{95} or His^{93} were deleted. The findings indicated that these mutants have no LH- or FSH-like activities. This proves that amino acids Lys^{95} and His^{93}, in particular, and the COOH-terminal amino acids in the α-subunit, in general, are extremely important in the interaction with LH- and FSH receptors (Jeoung et al., 2010).

When producing recombinant proteins from CHO-K1 cell lines containing eCG mutant genes, the changed amino acid Asn^{56} of N-eCG insignificantly alters the production of the recombinant protein (Min et al., 1996). The same outcomes were revealed in other studies that deleted the oligosaccharide chains at Asn^{56} and CTP of sc-eCG. However, the replacement of Asn^{82} of the α-subunit and Asn13 of the β-subunit led to a remarkable decrease in the secretion of this hormone into the culture medium. These results suggest that the N-linked saccharides at Asn^{13} and Asn^{82} have a vital function in the protein secretion of the CHO-K1 cell line (Min et al., 2019). In another research, the O-linked glycosylation sites at the CTP in sc-eCG were removed, causing the protein production to be delayed for a few days after the transfection of the CHO-S cell line. Consequently, the O-linked saccharides of the CTP are necessary to produce the eCG glycoprotein (Byambaragchaa et al., 2021a; Lee et al., 2021). When the amino acids 94 - 96 of the β-subunit in the sc-eCG were changed, the amount of the produced recombinant protein and the elimination half-life of these mutant eCGs were similar to the sc-eCG WT (Park et al., 2010; Byambaragchaa et al., 2021b). Nevertheless, modification of amino acids 102 - 104 led to a great decrease in the amount of the secreted sc-eCG (Byambaragchaa et al., 2021b). In another study, when poly-histidine was added at the C- or N-terminus of the α or β-subunit in the heterodimeric recombinant eCG, the quantity of the eCG mutants produced in the baculovirus-Sf9 system was tripled compared to the sc-eCG WT, and it also reduced progesterone production compared to natural eCG but did not affect ovarian development (Legardinier et al., 2005).

CONCLUSIONS

Many studies on the production of recombinant eCG have been published because of the need of this product on the market to address the shortage of natural eCG (PMSG) (Table 1). However, there is still no commercial recombinant product that can be optimally effective in mammals. Further research is thus needed to expand our knowledge in this area and develop an effective new rec-eCG.
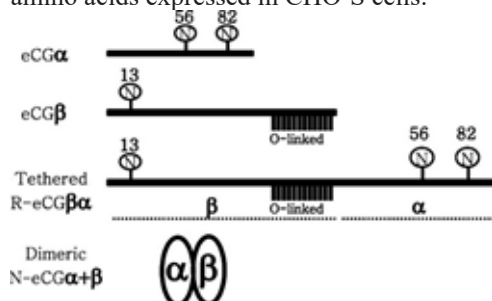
Table 1 The composition and nutrient levels of the diet.

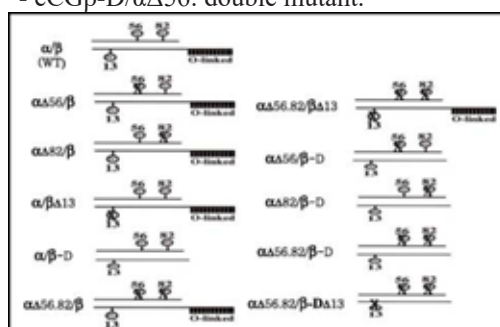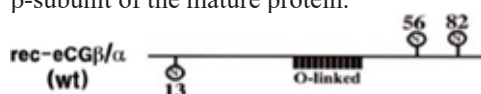| Structure of recombinant eCG	Biological Activity	References
- eCGβ/αΔ56: substitution of α-subunit (Asn56) N-linked glycosylation site; - eCGβ-D/α: removal of the O-linked glycosylation sites at the β-subunit; - eCGβ-D/αΔ56: double mutant. 	The cAMP responsiveness of eCG is strongly affected by the removal of N- and O-linked glycosylation sites in cells expressing eLH/CGR.	Byambaragchaa et al., 2021a; Lee et al. 2021.
Tethered R-eCG mutant was linked to the C-terminal of the β-subunit without signal peptide of the α-subunit composed of 24 amino acids expressed in CHO-S cells. 	Tethered R-eCG mutant can induce ovulation in mice.	Min et al., 2020.
- eCGβ/αΔ82: mutations in glycosylation site at Asn82 of the α-subunit; - eCGβΔ13/α: mutations in glycosylation site at Asn13 of the β-subunit; - eCGβ/αΔ56: deglycosylation site at Asn56 of the α-subunit; - eCGβ-D/α: removal of the C-terminal region of the β-subunit; - eCGβ-D/αΔ56: double mutant. 	Ovulation rates are not affected by rec-eCGβ/α derivatives and the percentage of nonfunctional oocytes produced by rec-eCGβ/α derivatives -treated mice is much lower.	Min et al., 2019.

| Structure of recombinant eCG	Biological Activity	References
rec-eCGβ/α: The epitope, myc-tag (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) was inserted into the tethered eCGβ/α between the first and second amino acids in the β-subunit of the mature protein.	rec-eCGβ/α did not increase cAMP responsiveness in the cells expressing eelFSHR.	Byambaragchaa et al., 2018.
Wild type eCG (eCGβ/α) and mutant eCG (eCGβ/αΔ56) have an N-linked oligosaccharide at Asn56 of the α-subunit.	Deglycosylation at Asn56 of the α-subunit of rec-eCGβ/α mutants inhibited internalization of agonist in CHO-K1 cells.	Park et al., 2017.
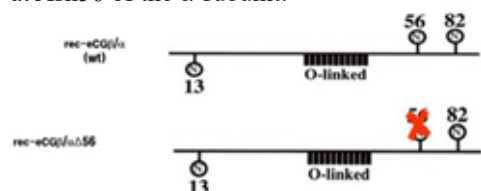
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AUTHOR CONTRIBUTIONS

Conceptualization and study design: T.M.D.N. and Y.C.; Manuscript drafting: D.T.T. and T.M.D.N.; Writing-review and editing: B.K.C., H.V.T., V.V.C., N.K.K., N.T.P.H. and N.V.H.; Manuscript finalization: T.M.D.N. and Y.C.

CONFLICT OF INTEREST

We have no conflict of interest.

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