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Preparation, characterization and application of long-acting FSH analogs for assisted reproduction

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ABSTRACT

Assisted reproduction technologies are widely used in humans and domestic animals and often include follicle stimulating hormone (FSH) in the protocol. One limitation with most of the available FSH preparations is the relative short half-life in the circulation that dictates multiple daily injections for the desired follicle development and superovulation. The development of bioactive long-acting structurally modified FSH analogs is desirable for human and veterinary use. In addition, optimal preparations and/or formulations are expected to improve the regimen and efficiency of the treatment. This review briefly describes the approaches that have been explored to extend the half-life of FSH in the circulation. These include strategies to increase the mass and/or charge of FSH and to prevent the dissociation of the hormone to inactive subunits components. Most of these strategies, except one that led to a registered drug (Elonva) indicated for controlled ovarian stimulation protocols in humans, are still in experimental stage.

1. Background and general considerations

Follicle stimulating hormone (follitropin; FSH) is widely used in assisted reproduction protocols in domestic animals and humans. FSH is a member of the glycoprotein hormone family that includes the gonadotropins -FSH, luteinizing hormone (lutropin; LH) and chorionic gonadotropin (CG)- and thyroid stimulating hormone (thyrotropin; TSH) [1]. Each hormone is a non-covalent heterodimer composed of a common α subunit and a hormone specific β subunit that determines receptor specificity [1] (Fig. 1). Only the heterodimer binds and activates the cognate receptor, and the individual subunits do not have a known activity. Together with LH, FSH is critical for successful ovulation as best shown by inactivating mutations in the gonadotropin subunits, and these mutated gonadotropins cause sub- or infertility [2].

FSH binds and activates its receptor that is classically located in the Sertoli cells in the testes, and in the granulosa cells in the ovaries [3,4]. FSH controls the development, growth, maturation and steroidogenesis in these cells and has a pivotal role in gamete production [3,5,6]. Accordingly, FSH is used to improve folliculogenesis to assist or enhance reproduction when desired. One limitation of using either native or recombinant (r) FSH preparations is the rather short-half life that usually dictates daily injections for multiple days in each cycle [7]. This review will briefly describe strategies developed- or predicted as exploitable to lengthen the $t_{1/2}$ of FSH. Hence, the generated long-acting FSH analog would thus improve the treatment regimen as related to the convenience and possibly the cost-effectiveness and outcome. These strategies include a) adding protein domains with determinants that elongate the $t_{1/2}$ in the circulation; b) introducing additional glycosylated peptides to modify the charge and increase the mass of the protein and accordingly reduce elimination from the body, c) PEGylating the hormone and d) preventing hormone disassembly (i.e., subunit dissociation) to stabilize the bioactive state of FSH by converting the non-covalent to a covalent heterodimer or into a single polypeptide chain that contains α and β subunit domains.

1.1. The biosynthesis and action of the gonadotropins

The gonadotrope cells of the anterior pituitary secrete FSH and LH in response to pulses of the hypothalamic decapeptide GnRH [1,2,8]. In the ovaries and testes LH and FSH regulate the production and release of sex steroids and coordinate crucial processes in reproduction like follicle maturation, ovulation, and

Abbreviations: CTP, carboxyl-terminal peptide; CHO, Chinese hamster ovary; WT, wild type; r, recombinant; h, human; bo, bovine; p, porcine; e, equine.

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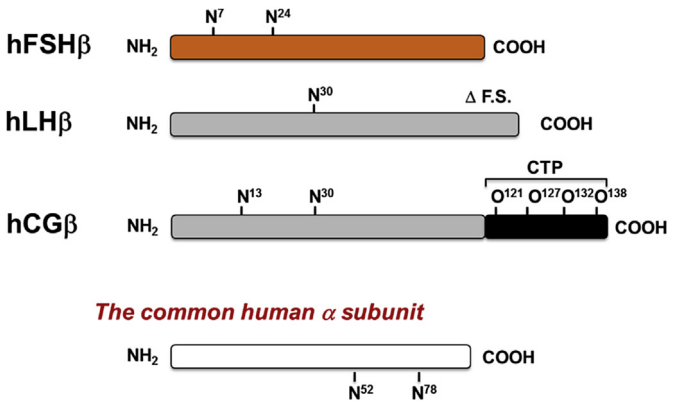

Fig. 1. The gonadotropin subunits. A schematic presentation of the human (h) gonadotropin specific β subunits and the common α subunit. The amino- and carboxy-terminal end of the protein and the location of the N- and O linked glycans are labeled. The numbers indicate the positions of the glycosylated amino acids. The hCGβ gene presumably evolved from the ancestral hLHβ gene, following gene duplication and a deletion (Δ) in the C-terminus region. This resulted in a frame shift (F.S.) and the elongation of the reading frame to create the O-glycosylated carboxy-terminal-peptide (denoted as CTP; black box) (see text for further details). Each β subunit can combine with the common α subunit to assemble a bioactive heterodimer.

spermatogenesis [2–5,9]. In primates (including humans (h)) and equids including horses (e)) an additional gonadotropin named chorionic gonadotropin (CG) is expressed in the placenta. CG binds and activates the LH receptor (LHR) and is required to delay luteolysis of the corpus luteum of pregnancy in primates [1].

The gonadotropin receptors are G protein-coupled receptors (GPCRs), predominantly expressed in the gonads, though their presence in other tissues was also shown [2–5,9–12]. The FSH receptor (FSHR) and the LHR are primarily coupled to the G_s/adenylyl cyclase/cAMP/PKA signal transduction machinery, and CREB is the major transcription factor transmitting the information to the nucleus [3,4,6,9,13,14]. Many reports indicate that phospholipase C (PLC) and phosphoinositide (PI) turnover, and various kinases, such as MAPKs, PI3K and Akt, are activated and that cascades in addition to the protein kinase A (PKA) pathway are involved in gonadotropin action, though their physiological role is less clear than that of PKA [15–23]. A coordinated activity of local factors, such as activin and insulin-like growth factor-1 (IGF-1) synergize with the gonadotropins, to modulate the gonadotropin action in the ovary, particularly on follicular growth [24–27]. This concerted activity, together with the identified activities of EGF and TGFβ family members produced in the ovary, as well as proteins interacting locally with the FSH and LH receptors [27–29], indicates that a complex signaling network regulates gonadal function and includes endocrine as well as local autocrine/paracrine mediators that influence gonadotropin activity. This complicated tuning of ovarian function together with incomplete understanding of the reproductive physiology contribute to the non-optimal results of the current assisted reproduction protocols that often include FSH.

1.2. Limitations of 'classical' FSH-like compounds in fertility treatments

In clinical setting in humans multiple daily injections of urinary FSH preparations and, in the last two decade or so, recombinant FSH (rFSH) are used [7]. The recombinant preparations offer a combination of potency, purity and pathogen free hormone. The carbohydrates (particularly the terminal moiety sugar of the attached glycans) are highly important for the circulatory survival of the glycoprotein hormones. Usually sialic acid is a favorable moiety for persistence in the blood. Accordingly, when considering the production of rFSH for potential therapeutic applications, mammalian derived cells like Chinese hamster Ovary (CHO) cells are currently preferable over alternative expression systems that naturally do not efficiently synthesize sialylated complex glycoproteins (e.g., insect cells, bacteria, yeasts and plants). Suboptimal response or hyperstimulation are major drawbacks of either native or rFSH preparations.

For theriogenological purposes equine LH/CG (eLH/CG; also known as PMSG) is widely used for superovulation, mainly because of its FSH-like actions in non-equid species, the availability and the extremely long half-life that reduces the number of injections during the treatment [30,31]. However, hyperstimulation of the ovaries and eliciting an immune response, particularly in repetitive uses, are among the major animal welfare related concerns of using PMSG. Moreover, inconsistent results and a reduced efficacy of the hormone may be associated with PMSG when used in assisted reproduction protocols in domestic animals. Porcine and bovine FSH preparations (including rFSH) are also available for use in livestock. Although their overall attractiveness is typically not higher than that of PMSG for reasons that include sub-optimal outcome, and the need of multiple consecutive injections that is stressful to the animals. Within the scope of this review, part of the limited effectiveness and attractiveness of porcine and bovine FSH is viewed as a rather short half-life that requires multiple injections. For veterinary use, it is likely that the development of an analog that has an extended circulatory survival compared to pituitary-derived FSH though shorter than that of eLH/CG is expected to improve the outcome of the current superovulation protocols and to increase the cost-effectiveness. In addition, formulation that include as the diluent gelatin or aluminum hydroxide gels, polyvynylpyrrolidone and hyaluronan have been developed to achieve sustained release, in order to reduce the number of FSH injections in assisted reproduction procedures in cattle; these technologies will not be discussed here (for recent examples see Refs. [32–35] and references therein).

2. Outline of design and methodology

2.1. Long acting FSH-CTP from the laboratory to the clinics

The only long-acting FSH variant currently approved for use in assisted reproduction protocols in humans is FSH-CTP (Elonva; approved for fertility treatment in Europe (2010) and in additional countries). The work by Irving Boime and his colleagues was the basis for this drug development. Briefly, in 1992 Fares et al. published their work on enhancing the circulatory survival of FSH by genetically engineering an FSH analog that included a peptide derived from hCG (known as the CGβ carboxy-terminal-peptide; CTP) (Fig. 2) [36]. This naturally occurring peptide was known to contain determinants for the enhanced circulatory survival of hCG in comparison to the other glycoprotein hormones [1].

The CTP is a hydrophilic, flexible stretch of some 30 amino acid residues that contains multiple sugar residues attached to serine and threonine residues. It is naturally located at the carboxy end of the CGβ subunit in primates and of the LHβ and CGβ subunits of equids [1,30,37–40]. It is thought that the CGβ gene evolved from the ancestral LHβ and an elongation of the reading frame led to the incorporation of the CTP domain in the extended subunit [41,42] (Fig. 1). Unlike in humans where gene duplication occurred, LHβ and CGβ are the products of a single gene in equids expressed in the pituitary and placenta, respectively, and both have a CTP [41,42]. The CTP is a special protein domain among the glycoprotein hormones since it is heavily O-glycosylated and contains 4 mucin-type

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