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Expression of long-acting human follicle-stimulating hormone analog from Chinese hamster ovary cells and functional characterization

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ABSTRACT

Follicle-stimulating hormone (FSH) is the pituitary hormone responsible for testicular tubal development and ovarian follicular recruitment and maturation. Both recombinant and purified urinary FSH are currently used for ovulation induction in women undergoing assisted reproduction. In this report, we describe Chinese hamster ovary (CHO-K1) cell expression of a novel long-acting human FSH single chain analog consisting of the native α and β subunit. A simple but readily scalable column chromatography method was developed to highly purify the recombinant FSH analog. *In-vivo* bioactivity was evaluated by the ovarian and uterine histopathology examinations. Single *SC* injection of the recombinant FSH analog (62.5 μg/kg) induced an equivalent increase the number of antral follicle, compared with commercial FSH preparation Folltropin-V (0.5 g/kg, twice daily for 4 days). The histopathology examination of the ovary and uterus confirmed the efficacy of the FSH analog. These findings indicate that this recombinant FSH analog could serve as a long-acting FSH preparation.

KEYWORDS

Recombinant human FSH;
Hormone analog;
CHO.

INTRODUCTION

Infertility affects 10%–15% couples trying to conceive. Assisted reproductive technologies provide the highest chance of success for those couples[1]. Human gonadotropins have been used to treat infertility for several decades and exogenous follicle-stimulating hormone (FSH) remains a key element of modern ovarian stimulation techniques[2]. Current FSH medications require a subcutaneous injection on a daily or twice-daily schedule for as long as 10-12 days per assisted reproduction cycle. Daily injections of human FSH are not only inconvenient, but also possibly contribute to the stress perceived by the patients[3]. Obviously, fewer injections may lessen the complexity and psychological burden.

Several different approaches have been undertaken to generate long-acting FSH preparations[4]. An elegant approach has been previously developed by fusing the carboxy terminal peptide (CTP) of the β subunit of hCG to native human FSH[5]. A single injection of the CTP-containing FSH can replace daily FSH injections for the first week of ovarian stimulation for in vitro fertiliza-

tion[6-11].

In our study, we developed a novel long-acting human FSH analog, by fusion of the native α and β subunit of human follicle-stimulating hormone together with a specific linker. The human FSH analog was expressed with CHO-K1 cells under serum-free conditions. The effect of this newly developed FSH analog on folliculogenesis and ovulation in rats was investigated.

MATERIALS AND METHODS

Materials

CHO-K1 cells (ATCC, CCL-61); Cell culture media and reagents (Invitrogen, USA); Rabbit anti-FSH antibody and Got anti-rabbit secondary antibody (Abcam Biosciences, UK); Folltropin-V (Bioniche, Canada); FSH ELISA Kit (Abnova, Taiwan).

Construction of a recombinant FSH analog expression vector

The eukaryotic expression vector pcDNA3.1 containing the ampicillin, neomycin resistance genes, and a strong promoter of the cytomegalovirus was used. An FSH analog expression frame that encodes α and β subunits of human FSH was inserted into the vector. The α and β subunits were conjoined by a linker sequence encoding a 19 amino acid polypeptide with two N-linked glycosylation signal sequences (Asn-Ala-Thr).

Characterization of the purified FSH Analog

The purified recombinant FSH analog was identified using FSH ELISA kit, according to the manufacturer's instructions, and Western Blotting. SDS-PAGE was performed to analyze the purity. Its biopotency was determined using the traditional Steelman-Pohley assay[12].

Histopathology examinations

Specimens of the ovary and uterus were fixed in 10% formaldehyde, processed using routine histology procedures, embedded in paraffin, cut in 5μm sections and mounted on a slide. The samples were stained with hematoxylin and eosin (H&E staining) for histopathology examination.

Statistical analysis

The significance of the differences between the experimental and control groups was tested by one-way analysis of variance and was considered to be significant for values of $P<0.05$. Post hoc analysis was carried out using Duncan multiple range tests.

RESULTS

Design of FSH analog mammalian expression vectors

A mammalian expression vector for recombinant FSH analog was constructed (Figure 1). A sequence that coding FSH analog was synthesized; a linker sequence was inserted to join the β and α subunits together. The linker contains a consensus sequence for the addition of N-linked carbohydrates.

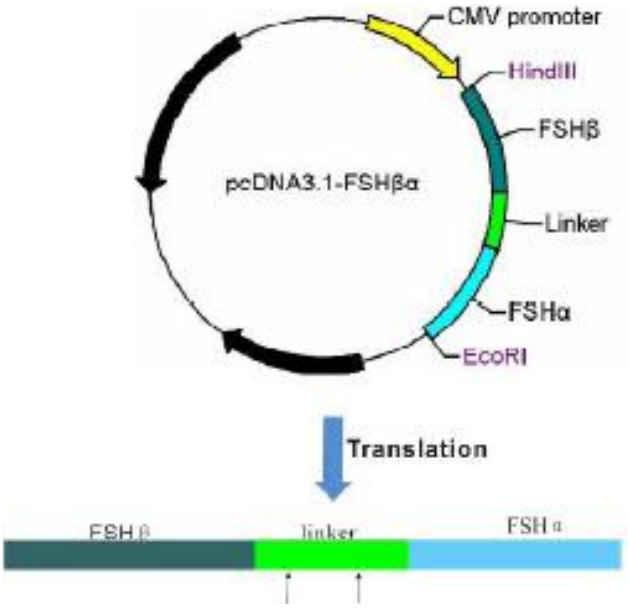

Figure 1: Vector map of FSH analog expression vector pcDNA3.1–FSHβα. The potential N-linked glycosylation sites (↑) are indicated on the linker sequence.

Expression of FSH analog in CHO-K1 cells

We established a stable single clone that expressed FSH analog at a level of 2.5 mg/L. The established clone was grown in a 10 liter bioreactor suspension culture without FBS.

Production and analysis of FSH analog

Recombinant FSH analog was purified from the culture supernatant with the column chromatography. The purified proteins were concentrated and buffer exchanged into PBS using ultrafiltration spin columns, and subse-

quently quantified using an ELISA assay. From the Coomassie blue-stained gel (Figure 2A), we noted a major thick band that spanned from 49 to 55 kDa, the purity was less than 98%. The results of Western blotting for FSH (Figure 2B) suggested that the recombinant FSH analog protein was the major product in the purified bulk.

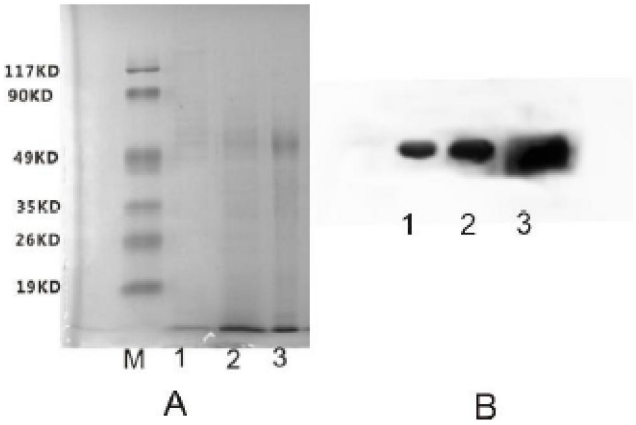

Figure 2 : Characterization of the purified FSH analog by SDS-PAGE (A) and Western Blotting (B). M: Protein molecular marker; lane 1, culture supernatant; lanes 2–3, elute from hydrophobic interaction and ion exchange chromatography, respectively.

Histologic analysis recombinant FSH analog effects

Representative sections from each group post treatment are shown in Figure 3. Histologic analysis revealed that both the lowest dose of FSH analog (2.5 μg/kg) and a single dose of Folltropin-V (0.5 g/kg) groups contained a number of smaller preantral follicles with some larger preantral follicles occupying the periphery of the ovary, whereas the higher dose of FSH analog (12.5 and 62.5 μg/kg) and the multiple doses of Folltropin-V groups contained a uniform distribution of early antral follicles.

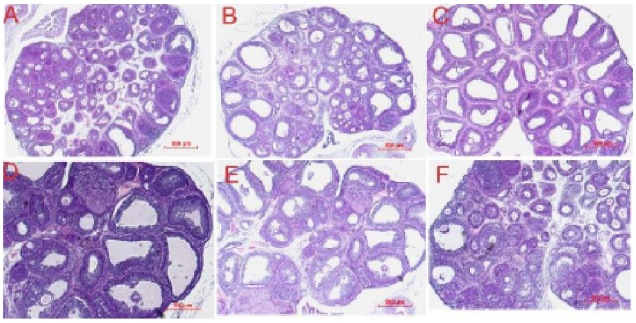

Figure 3 : Reprehensive photomicrographs of H&E-stained ovarian sections (4×). A: Saline; B: FSH (2.5 μg/kg); C: FSH (12.5 μg/kg); D: FSH (62.5 μg/kg); E: Folltropin-V (multiple doses, 0.5 g/kg/dose); F: Folltropin-V (single dose, 0.5 g/kg)

Folltropin-V (multiple doses, 0.5 g/kg/dose); F: Folltropin-V (single dose, 0.5 g/kg)

In the higher dose of FSH analog (12.5 and 62.5 μg/kg) and the multiple doses of Folltropin-V groups, uterine horns were found dilated and vascularized, endometrium surface epithelium was converted from cuboidal to columnar and irregular and folded structure was observed in Figure 4. In controls, the endometrium was found cuboidal and smooth. There were no obvious changes in the lowest dose of FSH analog (2.5 μg/kg) and a single dose of Folltropin-V (0.5 g/kg) groups, compared with the control.

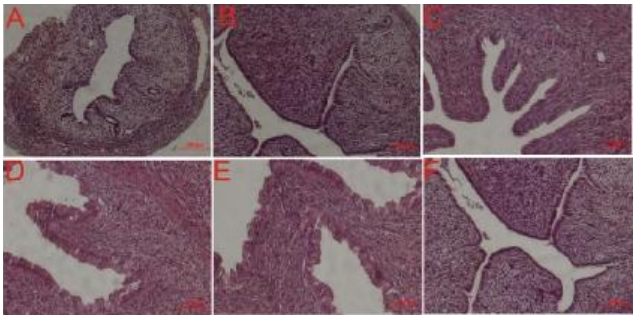

Figure 4 : Reprehensive photomicrographs of H&E-stained uterine sections (4×). A: Saline; B: FSH (2.5 μg/kg); C: FSH (12.5 μg/kg); D: FSH (62.5 μg/kg); E: Folltropin-V (multiple doses, 0.5 g/kg/dose); F: Folltropin-V (single dose, 0.5 g/kg)

DISCUSSION

The relatively short terminal half-life and rapid metabolic clearance of current FSH preparations requires that daily injections are administered to maintain steady state FSH levels above the threshold level during ovarian stimulation. Frequent injections may increase stress, error rates and the treatment burden experienced by IVF patients[10,13].

Remodeling of carbohydrate complexity can significantly affect the biological activity of glycosylated proteins[14,15]. A long-acting FSH, corifollitropin alfa, that employs a CTP together with the carboxyterminus of FSH β subunit is currently marketed to provide a more convenient dosing schedule for clinical use[16]. The CTP contains a series of four tightly spaced O-oligosaccharides, which are presumed to be responsible for delaying hormone metabolism in vivo[14]. In this report, we developed a long-acting recombinant FSH analog containing additional N-linked carbohydrate on the linker,

which joined the and β subunit of human FSH into a single chain. The ability of our recombinant FSH analog obtained from the serum-free culture medium of CHO cells on induction of folliculogenesis in rats was confirmed. An obvious advantage of our FSH analog is the potential for a greatly reduced dosing frequency. The efficacy of single dose of our FSH analog was equal to commercially available FSH preparation (Folltropin-V) that was administrated 8 times consecutively, in folliculogenesis and ovulation induction in rats. In addition, our FSH analog could be produced in bulk, so it's easy for scaled manufacture in the future.

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