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Abogada
LUZ CLEMENCIA DE PÁEZ

ASUNTO

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| Trámite | 11 |
| Evento | 378 |
| Actuación | 519 |
| Oficio | 10679 |
| Folios | 5 |

Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (Fase Nacional) ☒ Capítulo I ☒ Capítulo II ☐

No. Solicitud Internacional PCT/US2006/048898
Fecha de Presentación Internacional 21/Diciembre/2006

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

**1. Documentos estudiados**

| | | |
|---|---|---|
| Descripción: | Folios 34 a 66 | 22/Julio/2008 |
| | Folios 104 a 133 | 21/Octubre/2008 |
| Reivindicaciones: | Folios 67 a 69 | 22/Julio/2008 |
| | Folios 98 a 100 | 21/Octubre/2008 |
| | Folios 101 103 | 21/Octubre/2008 |
| Dibujos: | Folios 71 a 72 | 22/Julio/2008 |
| Listado de Secuencias: | Folios 73 a 77 | 22/Julio/2008 |
| Prioridad: | US 60/753,637 | 22/Diciembre/2005 |

**2. Análisis de la Prioridad**

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

**3. Objeto de la invención**

Título de la solicitud: "Mutantes de FSH".

El problema planteado en esta solicitud consiste en la necesidad de suministrar una hormona de estimulación de folículos (FSH) que pueda administrarse a intervalos menos frecuentes y que provea un nivel más estable de actividad FSH circulante.

Para resolver este problema técnico la solicitud en estudio proporciona mutantes de FSH.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 14/43.

**4. Excepción a la Patentabilidad** (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 16 – 18 no es patentable de acuerdo con el Art citado.

**5. De la Solicitud de Patente**

**Reivindicaciones** (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 2 – 4 no carecen de concisión porque utilizan las expresiones "un vector", "un vector de expresión", lo cual dificulta determinar la extensión del objeto que se busca proteger. Se recuerda al solicitante que un vector se caracteriza por su secuencia de polinucleótidos.

La reivindicación 8 no es clara cuando dice "(...) de acuerdo con la reivindicación 7 en donde cualquiera entre 0 a 6 residuos de asparagina son glicosilados", porque cada una de las SEQ ID NO: 3-5 contienen hasta 5 residuos de asparagina.

La reivindicación 11 no es concisa porque utiliza el término "una célula", lo cual dificulta determinar la extensión del objeto que se busca proteger. Se sugiere al solicitante definir el tipo de célula y que es capaz de glicosilar la proteína.

Se requiere al Solicitante hacer las aclaraciones necesarias.

**6. Determinación del Estado de la Técnica**

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Diciembre 22 de 2005, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

| Item | Documento | Título | Fecha de Publicación |
|---|---|---|---|
| D1 | WO2005020934 | FSH glycosylation mutant | 10/Marzo/2005 |
| D2 | WO0158493 | Follicle stimulating hormones | 16/Agosto/2001 |

D1 http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20050310&CC=WO&NR=2005020934A2&KC=A2 divulga un FSH mutante donde la subunidad-beta comprende la SEQ ID NO: 4 y que puede estar glicosilado en residuos de asparagina para aumentar el tiempo de vida media (Resumen y Pág. 4, párrafo 17)

D2 http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20010816&CC=WO&NR=0158493A1&KC=A1 divulga un FSH mutante en las subunidades alfa y beta, donde ciertos residuos se han cambiado

para crear sitios de glicosilación para mejorar la vida media, comparada con FSH humana (Resumen y Pág. 58, ejemplo 9).

## 7. **Examen de Patentabilidad** (Art 14 D486)

**Nivel Inventivo** (Art18 D486)

La reivindicación 7 consiste en una FSH mutante, en donde la subunidad-beta comprende la secuencia de ID NO: 3, y en donde la subunidad-alfa comprende una secuencia codificada por el ácido nucleico de acuerdo con la reivindicación 1.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

| Características **esenciales** | D1 | D2 |
|---|---|---|
| Una FSH mutante, en donde:<br>a) la subunidad-beta comprende la secuencia de ID NO:3, y | Una FSH mutante, en donde la subunidad-beta comprende la SEQ ID NO:4, y<br><br>(Pág. 4, párrafo 17) | Una FSH mutante en la subunidad-beta, y<br><br>(Resumen y Pág. 58, ejemplo 9) |
| b) en donde la subunidad-alfa comprende una secuencia codificada por el ácido nucleico de acuerdo con la reivindicación 1. | Donde la subunidad-alfa comprende sitios de glicosilación<br><br>(Pág. 4, párrafo 17) | Mutante en la subunidad-alfa |

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 7 porque:

D1 divulga un FSH mutante donde la subunidad-beta comprende la SEQ ID NO: 4 y que puede estar glicosilado en residuos de asparagina para aumentar el tiempo de vida media (Resumen y Pág. 4, párrafo 17). La SEQ ID NO: 4 de D1 tiene 100% de identidad con la SEQ ID NO: 3 de la presente solicitud:

```
Sequence type explicitly set to Protein
Sequence format is Pearson
Sequence 1: WO2005020934 - SEQ ID NO: 4       111 aa
Sequence 2: 08-75688 - SEQ ID NO: 3           111 aa
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score:  100
```

D1 igualmente divulga que la actividad de la FSH mutante es comparable con la FSH humana y que la vida media del péptido mutante se mejora (Pág. 24, ejemplo 11). D1 también enseña un método para producir una mutante de FSH que comprende proveer una célula que comprende el ácido nucleico que codifica la FSH mutante y cultivar la célula (Pág. 5, párrafo 26) y una composición farmacéutica que comprende la FSH mutante (Pág. 6, párrafo 27).

La diferencia entre la invención, definida en la reivindicación 7 y la FSH mutante de D1 consiste en la secuencia de la subunidad-alfa.

D2 divulga un FSH mutante en las subunidades alfa y beta, donde ciertos residuos se han cambiado para crear sitios de glicosilación para mejorar la vida media, comparada con FSH humana (Resumen). D2 igualmente enseña un mutante FSH donde un fragmento que codifica la secuencia fue insertada corriente arriba de la

secuencia de la subunidad-alfa, incorporando dos sitios adicionales de glicosilación y que incrementa la vida media del mutante FSH, comparada con la FSH humana (Pág. 58 y 59, ejemplo 9). D2 también enseña un método para producir una mutante de FSH que comprende proveer una célula que comprende el ácido nucleico que codifica la FSH mutante y cultivar la célula (Pág. 66, reivindicación 37) y una composición farmacéutica que comprende la FSH mutante (Pág. 4, líneas 26-29).

La diferencia entre la invención, definida en la reivindicación 7 y la FSH mutante de D2 consiste en la secuencia de la subunidad-alfa.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a las FSH mutantes reclamadas en esta solicitud porque no parecen presentar una mayor actividad FSH y un mejor tiempo de vida media, según lo establecido en la descripción.

El Problema Técnico Objetivo de esta solicitud consiste en la necesidad de mutantes FSH alternativas a las ya conocidas en D1 y D2.

Teniendo en cuenta que D1 ya divulgaba un FSH mutante donde la subunidad-beta comprende la SEQ ID NO: 4, que tiene una identidad del 100% con la SEQ ID NO: 3 de la presente solicitud y que puede estar glicosilado en residuos de asparagina para aumentar el tiempo de vida media, con una actividad comparable con la FSH humana (Resumen, Pág. 4, párrafo 17 y Pág. 24, ejemplo 11), y que D2 muestra que sitios adicionales de glicosilación en la porción N-terminal de un mutante FSH incrementa la vida media del mutante FSH, comparada con la FSH humana (Pág. 58 y 59, ejemplo 9), la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, incluir sitios adicionales de glicosilación en la porción N-terminal de la subunidad-alfa de una FSH mutante, para obtener más péptidos con la misma actividad y con una mejorada vida media. La persona del oficio medianamente versada en la materia se vería motivada a incluir sitios adicionales porque es bien conocido en el estado del arte que la glicosilación de una extensión N-terminal prolonga la vida media e incrementa la actividad FSH *in vivo*[1,2].

De manera que la solución propuesta por esta solicitud en la reivindicación 7 y sus dependientes 8 – 10, y en las reivindicaciones 1 – 6 y 11 – 15 es obvia y no tiene nivel inventivo.

## 8. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

| Ítem | Documento | Reivindicaciones afectadas | Requisito que afecta |
|---|---|---|---|
| D1 | WO2005020934 | 1 – 15 | Nivel Inventivo |
| D2 | WO0158493 | 1 – 15 | Nivel Inventivo |

1. S. Perlman, *et al.* Glycosylation of an N-terminal extension prolongs the half-life and increases the *in vivo* activity of follicle stimulating hormone. The Journal of Clinical Endocrinology & Metabolism, Vol. 88. Pág. 3227-3235.
2. C. Weenen. Long-acting follicle-stimulating hormone analogs containing N-linked glycosylation exhibited increased bioactivity compared with O-linked analogs in female rats. The Journal of Clinical Endocrinology & Metabolism, Vol. 89, Pág. 5204-5212.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta a este requerimiento y presentarla por escrito

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

**JOSÉ LUIS SALAZAR LÓPEZ**
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 20 JUN. 2012

Elaborado por: Juan David Moncaleano
Revisado por: Consuelo Leguizamón

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# Glycosylation of an N-Terminal Extension Prolongs the Half-Life and Increases the *in Vivo* Activity of Follicle Stimulating Hormone

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**FSH is a key component in assisted reproductive technologies. Because of rapid clearance of the hormone, patients have to be treated with daily injections. To address this problem, a long-acting FSH mutein was created by introduction of additional N-linked glycosylation into the molecule. New glycosylation sites were introduced by two different approaches: structure-aided, site-directed introduction of sites within the FSH molecule and addition of N-terminal extensions. A mutein with the extension sequence ANITVNITV at the N terminus of the α-chain (FSH1208) was efficiently glycosylated at both new sites. This resulted in a molecule with increased size and charge, factors known to reduce renal clearance of proteins. FSH1208 was found to have a 3- to 4-fold increased serum half-life, compared with wild-type recombinant FSH. Furthermore, in spite of a lower *in vitro* activity, FSH1208 had a markedly increased *in vivo* potency, as shown by increased ability to augment the ovarian weight and stimulate the serum estradiol levels in rats. These characteristics make FSH1208 a possible candidate for improved infertility treatment. (*J Clin Endocrinol Metab* 88: 3227–3235, 2003)**

FSH IS A KEY REGULATOR OF GONADAL function. In men, the hormone is important for spermatogenesis (1, 2), and FSH is responsible for the growth and maturation of the ovarian follicles in women (3). Today FSH is a key component of modern treatment of infertility (4–6). FSH is used in assisted reproductive technologies for treatment of various anovulatory conditions including polycystic ovary syndrome and stimulation of multiple follicles in relation to for instance *in vitro* fertilization. The FSH products available today are either purified from human urine or recombinantly produced human (h) wild-type FSH and are, thus, very similar to naturally occurring FSH. FSH is administered by sc injections either by the physician or the patients themselves. The compounds have to be given daily throughout the treatment period because of a relatively short *in vivo* half-life. The daily injections and related local skin reactions are the most common inconveniences associated with the treatment.

FSH is a glycoprotein composed of two subunits, termed α and β. The α-subunit of FSH is identical to the α-subunit of other glycoprotein hormones [*i.e.* chorionic gonadotropin (CG), LH, and TSH], whereas the β-subunit is unique. The α-chain consists of 92 amino acid residues and has two N-glycosylation sites located at asparagine residues N52 and N78. It is noncovalently linked to the FSH β-chain composed of 111 amino acids. The β-chain also contains two N-glycosylation sites, located at residues N7 and N24 (7).

An important mechanism for elimination of FSH is through glomerular filtration (8). It is well known that FSH exists in many different isoforms because of differences in the glycosylation pattern and, hence, in the sialic acid content (7, 9). Isoforms with high content of sialic acid have a low isoelectric point, are negatively charged at physiological pH, and are not as easily filtered by the glomeruli. Thus, the isoforms with a high sialic acid content remain longer in circulation and have been shown to have a higher *in vivo* activity (10–12).

Previously a chimeric FSH-hCG molecule created by fusion of the FSH β-chain with a sequence of the C terminus of hCG has been constructed. This molecule, which has a higher molecular weight than wild-type FSH because of the presence of O-glycosylation in the extension, was shown to have increased half-life and *in vivo* activity (13–16).

The goal of the present study was to develop an FSH-based molecule with retained *in vitro* and *in vivo* action and prolonged *in vivo* half-life, which would allow reduction of the injection frequency and, thus, increase patient convenience and compliance. The strategy was to increase the molecular weight and the charge of the molecule by introduction of additional glycosylation and, thus, reduce glomerular filtration.

Two different approaches were used for introduction of extra glycosylation sites. One was based on identification of areas within the native structure of the FSH molecule in which glycosylation sites potentially could be introduced without distortion of receptor binding and the overall tertiary structure. The second approach was based on N-terminal elongation using sequences encompassing glycosylation sites. Several molecules with increased glycosylation were identified through both strategies and one of these, termed FSH1208, was selected for further *in vitro* and *in vivo*

Abbreviations: CG, Chorionic gonadotropin; CHO, Chinese hamster ovary; EPO, erythropoietin; FBS, fetal bovine serum; IEF, isoelectric focusing; h, human; rFSH, recombinant FSH.

studies to substantiate that it could form the basis for a new, improved infertility treatment.

## Materials and Methods

### *FSH structural analysis*

A series of 50 models of the three-dimensional structure of hFSH was built based on two available hCG structures (17, 18) using the program Modeler 98 (MSI Inc., San Diego, CA). In the α-chain four N-terminal residues (A1, P2, D3, and V4) and three C-terminal residues (H90, K91, and S92) were not modeled because they are not identified in the hCG structures. All of the hFSH-β-chain was modeled, even the part that has no homologous residues in the hCG structures. The accessible surface area was calculated for each of the 50 model structures using the computer program Access, version 2 (Yale University, New Haven, CT). Although this work was in progress, a structure of an hFSH variant was published (19). The accessible surface area based on this structure generally confirmed the calculation based on the model structures.

### *Construction of plasmids for expression of FSH*

Genes encoding the human FSH α- and β-subunits were constructed by assembly of synthetic oligonucleotides using PCR. The native signal sequences were maintained, except for a mutation in the β-subunit at position 2 (Lys to Glu). The codon usage of the genes was optimized for high expression in mammalian cells using information on *Homo sapiens* codon usage from http://www.kazusa.or.jp/codon/ (20), at the time based on 12,512 coding sequences.

The pcDNA3.1(+)/Hygro (Invitrogen, Carlsbad, CA) and pcDNA3.1(+)/Zeo (Invitrogen) were used as expression vectors. An intron from pCI-Neo (Stratagene, La Jolla, CA) was added to further optimize the protein production. The intron was amplified from pCI-Neo using 5′-CCGTCAGATCCTAGGCTAGCTTATTGCGGTAGTTTATCAC-3′ and 5′-GAGCTCGGTACCAAGCTTTTAAGAGCTGTAAT-3′ as primers. The PCR product was subcloned into pcDNA3.1(+)/Hygro using NheI and HindIII, yielding PF033. The intron was transferred to plasmid pcDNA3.1(+)/Zeo by replacing a 758-bp MluI-XhoI fragment in the plasmid with the intron-containing MluI-XhoI fragment from PF033, resulting in pBvdH957. Using BamHI and XbaI, the synthetic α-gene was subcloned into PF033 and the β-gene into pBvdH957, resulting in pBvdH977 and pBvdH1022, respectively. Mutant forms of FSH-α and -β were generated by PCR-based site-directed mutagenesis using pBvdH977 and pBvdH1022 as templates. The sequences of all constructs were verified by DNA sequencing. Plasmids containing both the α- and β-encoding genes were generated by subcloning FSH-α containing NruI-PvuII fragments from pBvdH977 (or derivatives) into pBvdH1022 (or derivatives) linearized with NruI.

### *Expression of FSH in CHO cells*

FSH was expressed in Chinese hamster ovary (CHO) K1 cells (CCL-61; ATCC, Manassas, VA). Cells were grown in media (catalog no. 32571-028 or 31330-038; Life Technologies, Inc., Carlsbad, CA) containing 1:10 fetal bovine serum (FBS) (catalog no. 02-701F; BioWhittaker, Inc., Walkersville, MD) and 100 U/ml penicillin and 100 μg/ml streptomycin (pen-strep) at 37 C, 5% $CO_2$. Plasmids were transfected using Lipofectamine 2000 (Life Technologies, Inc.). Twenty-four to 48 h after transfection, culture media were collected for Western blotting. Stable clones expressing FSH were isolated by growth under selection pressure. The expression levels of individual clones were determined by ELISA. High producers were selected for large-scale FSH production: Cells were grown until confluence in 1700-$cm^2$ roller bottles containing 300 ml media (catalog no. 31330-038; Life Technologies, Inc.) with the same additives. The media were then changed to UltraCHO with L-glutamine (catalog no. 12-724Q; BioWhittaker, Inc.) with addition of 1:500 EX-CYTE VLE (catalog no. 81-129, Serological Proteins Inc., Kankakee, IL) and pen-strep. The media were replaced with fresh media after 48 h of growth. After another 48 h, the media was replaced with serum-free media (catalog no. 21041-025, with the addition of 1:500 ITS-A; catalog no. 51300-044, 1:500 EX-CYTE VLE and pen-strep; Life Technologies, Inc./BRL). Subsequently, every 24 h, culture media were harvested and replaced with 300 ml fresh serum-free media. Growth in roller bottles with daily harvests was continued for up to 10 d.

### *Purification and characterization of FSH*

Harvests from the CHO cells were concentrated by ultrafiltration (10-kDa cut-off membrane), pH was adjusted to 8.0, and the conductivity lowered to 1–2 mS/cm by dilution with distilled water. A diethylaminoethyl Sepharose (Pharmacia, Stockholm, Sweden) column was equilibrated with 16 mM ammonium acetate buffer (pH 8). Subsequently the sample was applied and FSH was eluted with 0.16 M ammonium acetate (pH 8). After addition of ammonium sulfate to a concentration of 1.5 M and adjustment of the pH to 7.0, the sample was applied on a butyl Sepharose (Pharmacia) column. After application, the column was washed with 1.5 M $(NH_4)_2SO_4$, 20 mM ammonium acetate (pH 7), and FSH was eluted with 20 mM ammonium acetate buffer (pH 7). Eluated FSH was dialyzed against 50 mM sodium phosphate, 150 mM NaCl (pH 7.2), using 12–14 kDa cut-off dialysis tubing. For the final step, an anti-FSH-β monoclonal antibody (RDI-FSH909, Research Diagnostics) was immobilized to CNBr-activated Sepharose (Pharmacia). One milligram antibody was coupled per milliliter resin. The immunoaffinity resin was equilibrated with 50 mM sodium phosphate, 150 mM NaCl (pH 7.2). After application of the sample, the column was washed with 50 mM sodium phosphate, 1 M NaCl (pH 7.2), followed by equilibration with 50 mM sodium phosphate, 150 mM NaCl (pH 7.2). Elution from the column was performed using 1 M $NH_3$, 40% (vol/vol) isopropanol, and the pH in the eluted fractions was immediately neutralized with 2 M acetic acid to pH 6–8. The purified FSH was concentrated and diafiltrated using Vivaspin 20 modules, 10-kDa cut-off membrane (Vivascience, Hannover, Germany), to 50 mM sodium phosphate, 150 mM NaCl (pH 7.2), and microfiltrated using a 0.22-μm filter. The purified protein was analyzed by SDS-PAGE under nondissociating conditions (without boiling). For storage at −80 C, BSA was added to 0.1% (wt/vol).

N-terminal sequencing was carried out following SDS-PAGE and electroblotting onto polyvinyl difluoride membranes using a 494 protein sequencer (PE Applied Biosystems, Foster City, CA). For amino acid analysis, lyophilized protein samples were hydrolyzed for 16 h at 110 C in 6 M HCl containing 1% phenol in $N_2$-blanketed glass vials before quantification of the liberated amino acids using the AccQTag system (Waters, Milford, MA).

Western blotting was performed using rabbit antihuman FSH (AHP519, Serotec) or mouse antihuman FSH-β (MCA338, Serotec, Raleigh, NC) as primary antibody, and an ImmunoPure Ultra Sensitive ABC peroxidase staining kit (Pierce Chemical Co., Rockford, IL) for detection.

Samples were separated on pH 3–7 isoelectric focusing (IEF) gels (Novex) for analysis of pH at the isoelectric point (pI). After electrophoresis, proteins were blotted onto Immobilon-P (Millipore Corp., Billerica, MA) membranes, and a Western blot was performed.

### *In vitro activity assay, ELISA, and specific activity*

FSH *in vitro* activity was measured using a reporter cell line expressing the hFSH receptor and a reporter gene (21, 22). This cell line was obtained as follows: CHO-K1 cells were transfected with a pcDNA3 (Invitrogen) derivative containing the FSH receptor cDNA (kindly supplied by Dr. A. Hsueh, Stanford University, Palo Alto, CA). Stable clones were isolated, and a clone that responded to FSH stimulation was found using a cAMP-SPA RIA (Amersham, Cardiff, UK). Subsequently a plasmid containing 6 cAMP-responsive elements upstream of the Firefly luciferase reporter gene was stably introduced into this clone. For measurement of FSH activity, the reporter cells were suspended in DMEM/F-12 (without phenol red) containing 1.25% FBS and seeded in white 96-well culture plates at a density of 15,000 cells/well (100 μl). After incubation overnight at 37 C, 5% $CO_2$, 25 μl sample or FSH standard (follitropin-β), diluted in media containing 10% FBS was added to each well. Subsequently the plates were incubated for 3 h, followed by addition of 125 μl LucLite substrate (Packard Bioscience, Groningen, the Netherlands). Finally, the luminescence was measured on a TopCount luminometer (Packard Bioscience) in single photon counting mode.

FSH concentrations were determined by ELISA (FSH EIA, DRG Instruments GmbH, Marburg, Germany). This ELISA kit has a reported minimal detection level of 1 mIU/ml (~0.1 ng/ml) and a coefficient of

variance of less than 6% within the measured range. A dilution series of follitropin-β (Puregon, Organon, Oss, The Netherlands) in PBS with 0.1% BSA was used as reference. A follitropin-β batch containing 400 IU [relative to international reference IS 70/45 (23)] per milliliter was used. It was assumed that the specific activity of this batch was 10,000 IU/mg, *i.e.* that the batch contained 40 μg FSH per ml (23). An amino acid analysis of an FSH1208 aliquot determined a protein concentration of 0.34 mg/ml, corresponding exactly to the concentration measured (0.34 mg/ml) in the ELISA assay. This demonstrated that the ELISA set-up was suitable for determination of concentrations of FSH1208.

The specific activity of FSH1208 was determined by measurement of the *in vitro* bioactivity using the luciferase assay, and measurement of the FSH content of the samples was determined by ELISA. The specific activities were assessed using follitropin-β as standard and expressed in international units (see above).

### *Animal studies*

Female Sprague Dawley rats were obtained from the breeder at a weight of 200–220 g for the pharmacokinetic studies and at an age of 21–22 d for the pharmacodynamic studies. They were housed in a temperature-controlled room at 23 C, with a 12-h light period from 0600 h to 1800 h and free access to food and water. All experiments were conducted in accordance with accepted standards of humane animal care, reviewed and accepted by the Animal Experimentation Inspectorate in accordance with the Animal Testing Act set by the Danish Ministry of Justice.

Pharmacokinetics were studied by injection of 10 μg protein either iv, in an injection volume of 250 μl, or sc in an injection volume of 500 μl. Preparations were formulated in a DPBS buffer (catalog no. BE 17-512F; BioWhittaker, Inc.) containing 0.1% (wt/vol) BSA (product no. A3059; Sigma, St. Louis, MO). In each experiment, three rats in the reference group received follitropin-β (Puregon; Organon), and three other rats received FSH1208. Blood samples were taken in a time span over 72 h and incubated at room temperature for 90 min. Serum was isolated by centrifugation (5000 rpm for 30 min) and stored at −80 C. Serum samples were analyzed for FSH content by ELISA.

Pharmacodynamics were determined by the ovarian weight augmentation assay (24) and measurement of estradiol levels in serum. All animals received a daily dosage of 13.3 U hCG (catalog no. C-1063; Sigma) and either FSH or a buffer vehicle. Injections had a volume of 200 μl and were given sc. The hCG was formulated in 0.01 M sodium phosphate (pH 7.2) containing 30 mg mannitol/ml, and FSH was formulated as described above. The first group of animals received vehicle on d 1 and 2; the second group received 0.25 μg (2.5 IU) follitropin-β on d 1 and vehicle on d 2; the third group received 0.25 μg follitropin-β both days; the fourth group received 0.25 μg (equivalent to about 0.6 IU) FSH1208 on d 1 and vehicle on d 2; the fifth group received 2.5 IU FSH1208 on d 1 and vehicle on d 2. In the 72-h studies, the groups and dosages were the same, but the rats in group 3 were stimulated for an extra day with follitropin-β, and all others received vehicle on d 3. Blood samples were withdrawn every 12 h in the 48-h setup. Serum samples were isolated, stored as described above, and analyzed for 17β-estradiol level using an ELISA kit (catalog no. RE-52041; IBL-Hamburg). The minimum detectable concentration of estradiol was reported by the provider to be 10 pg/ml, and the coefficient of variance was maximally 10% in the measured range. The animals were killed by cervical dislocation, and the ovarian weights were determined after decapsulation.

The results were analyzed using the computer program PRISM (version 3.01, 1999; GraphPad Software, Inc., San Diego, CA). Statistical significance of differences was assessed by ANOVA tests either one way or for repeated measures followed by Bonferroni's multiple comparison test. Probabilities less than 0.05 were considered significant.

## Results

### *FSH variants*

Two different approaches of introduction of additional glycosylation sites were applied in the current study. In one approach the structural model of FSH was used to identify suitable locations for introduction of mutations. The other approach used the model to extend the protein with sequences housing possible glycosylation sites.

The structural analysis of FSH was performed in an attempt to identify sites in the molecule in which N-linked glycosylation sites (Asn-X-Thr/Ser) could be introduced without significant distortion of the tertiary structure or the receptor binding of the molecule. Consequently, the structural analysis was focused on identifying sites that were located on the surface of the molecule distant from the presumed receptor-binding area (7, 25). Such possible N-glycosylation sites were chosen through identification of amino acid residues in which more than 50% of the side chain was exposed at the surface of the molecule, calculated as an average over the series of the 50 models of the three-dimensional structure of hFSH that were built. Furthermore, residues that had a proline residue at position +1 or + 3 were excluded because it is known that proline residues in these positions reduce the glycosylation efficacy of the asparagine residues (26). About 50 sites were identified in each of the two subunits in the series of models that were built. Subsequently residues that had a threonine or serine residue located at position +2 were identified because these could be transformed into glycosylation sites through exchange of only one amino acid residue. The α-chain housed four such possible N-glycosylation sites [E9(α)N, F17(α)N, R67(α)N, and H90(α)N], whereas five sites were identified in the β-chain [Y58(β)N, L73(β)N, S89(β)N, D90(β)N, and Y103(β)N]. Only three of the four possible exchanges were constructed in the α-chain because it is known that residues in the C-terminal part of the subunit, including H90(α), are important for receptor binding and signal transduction, and thus, mutation of this residue would most likely affect the function of FSH (25, 27). Similarly, S89 and D90 of the β-chain were excluded because it is believed that this part of the β-chain is also involved in the receptor binding and activation (7, 28).

An alternative approach was based on introduction of extra glycosylation sites by extension of the two N termini because they are located distant from the presumed receptor-binding site and because it is unlikely that an extension results in distortion of the tertiary structure. The extensions comprised four to nine amino acid residues including one or two Asn-X-Thr sites. Three different extensions were used: ANIT, ANITV, and ANITVNITV, all of which included amino acid residues with relatively small, nonpolar side chains (Ile, Val, AL) to minimize steric hindrance and 1–2 amino acids conserved in the extreme N terminus, compared with the wild-type chains.

All muteins were produced in CHO cells in different combinations, and Western blot analyses were performed to assess the degree of glycosylation. Examples of different degrees of glycosylation are shown in a Western blot in Fig. 1B. Lanes 2 and 4 show the muteins Y103(β)N and Y58(β)N, respectively. It is seen that only a small percentage of the Y103(β)N molecules has a higher molecular weight than the wild-type recombinant FSH (rFSH) in lane 1, indicating that only a minor fraction of the molecules was glycosylated at the introduced asparagine residue. In contrast, almost all molecules of the extension variant in lane 3, which has two additional glycosylation sites added to the N-terminal part

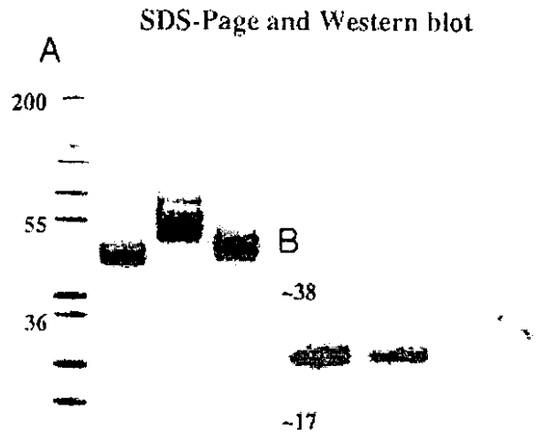


FIG. 1. A, SDS-PAGE of rFSH run under nondissociating conditions. Lane 1, Protein standard with mass markers from 14 to 200 kDa; lane 2, follitropin-β; lane 3, FSH1208, α-chain extension mutein with two additional glycosylation sites; lane 4, β-chain extension mutein (NSNITVNITV) with two additional glycosylation sites. B, Western blotting of SDS-polyacrylamide gel run under denaturing conditions. A β-subunit-specific monoclonal antibody was used. The changes in the molecular weights reflect the different degree of glycosylation of the different variants. Lane 1, Wild-type FSH (produced in our own laboratory); lane 2, FSH β variant, Y103N; lane 3, β-chain extension variant with two additional glycosylation sites (NSNITVNITV); lane 4, a point-mutated FSHβ variant.Y58N.

of the β-chain between residue 2 and 3 (NS NITVNITV CEL), were glycosylated, at least at one position in the extension.

Surprisingly, all of the muteins, in which glycosylation sites were introduced within the two chains, were either not glycosylated at all or only partially glycosylated. Generally, extensions muteins were glycosylated to a larger degree than the single point-mutated muteins. One such extension mutein, FSH1208, which had the sequence ANITVNITV added to the N terminus of the α-chain, was selected for further studies because this mutein was found to be glycosylated to a higher extent than any of the other variants.

### *Characterization of FSH1208*

*Expression and purification.* Stable CHO clones coexpressing the mutated α-subunits with the wild-type β-subunits were isolated and used for large-scale production. Expression yields were about 0.2–0.25 mg/l. FSH1208 was purified as described, resulting in 90–95% purity as judged by SDS-PAGE (Fig. 1A). SDS-PAGE, run under nondissociating conditions (Fig. 1A), showed wild-type, rFSH (follitropin-β) migrating as a band with an apparent mass of 45 kDa (lane 2), slightly diffuse because of heterogeneity in the attached carbohydrates. In comparison, FSH1208 migrated as a band with an apparent mass of 55 kDa (lane 3). The β-extension mutein (NSNITVNITV) seen in lane 4 has a molecular weight in between the weight of follitropin-β and FSH1208, indicating that this extension mutein is not as efficiently glycosylated as FSH1208.

The charge of the purified protein was analyzed by IEF. Recombinantly produced wild-type FSH (follitropin-β) was found to consist of a series of isoforms with a pI between 4.0 and 5.2, in accordance with the literature (29). In contrast, FSH1208 consisted of isoforms with a pI between 3 and 4.8 with the majority of the isoforms in the lowest part of the range (Fig. 2).

### *N-terminal sequencing and in vitro activity*

N-terminal sequencing of FSH1208 demonstrated the presence of the N-terminal extension on the α-chain. The sequence of the N-terminal β-chain was also identical to the known sequence of the wild-type β-FSH (30), although the first two residues were absent. This truncation has previously been found for both pituitary and rFSH (31–33). N-terminal amino acid sequence determination of three different purification batches of FSH1208 indicated that approximately 80% of N2 was glycosylated and more than 95% of N6 was glycosylated.

*In vitro* bioactivity assay showed that the specific activity of FSH1208, 2.3 ± 0.5 IU/μg (n = 11), was lower than that of rFSH, follitropin-β, 10 IU/μg (23).

### *Pharmacokinetic studies*

The pharmacokinetic profile of FSH1208 was determined either sc or iv by administration of a single dose of 10 μg/rat. Figure 3A shows the serum concentration of wild-type rFSH (follitropin-β) and FSH1208 after iv administration as measured by ELISA. The concentration of both proteins declined rapidly in the initial distribution phase, followed by a constant elimination at a slower rate. FSH1208 had a significantly longer *in vivo* half-life than follitropin-β. The terminal half-life for FSH1208 after iv administration was calculated to 22 h, and the terminal half-life for follitropin-β was calculated to 6 h, in accordance with previous reports (23). It was not possible to detect follitropin-β beyond the first 24 h.

The absorption into the circulation was slow after a single sc dosage of either of the FSH proteins (Fig. 3B). The maximal level of follitropin-β was reached after 12 h, whereas the concentration of FSH1208 continued to rise until 24 h after the injection. The half-life of follitropin-β after sc administration

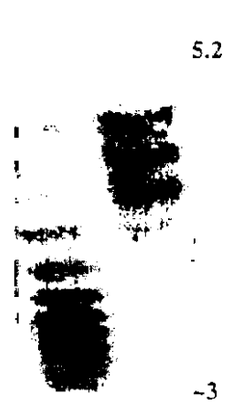


FIG. 2. IEF using pH 3–7 gels, followed by a Western blot analysis performed using a mouse antihuman FSHβ as primary antibody. IEP of FSH1208 (lane 1) and follitropin-β (lane 2).

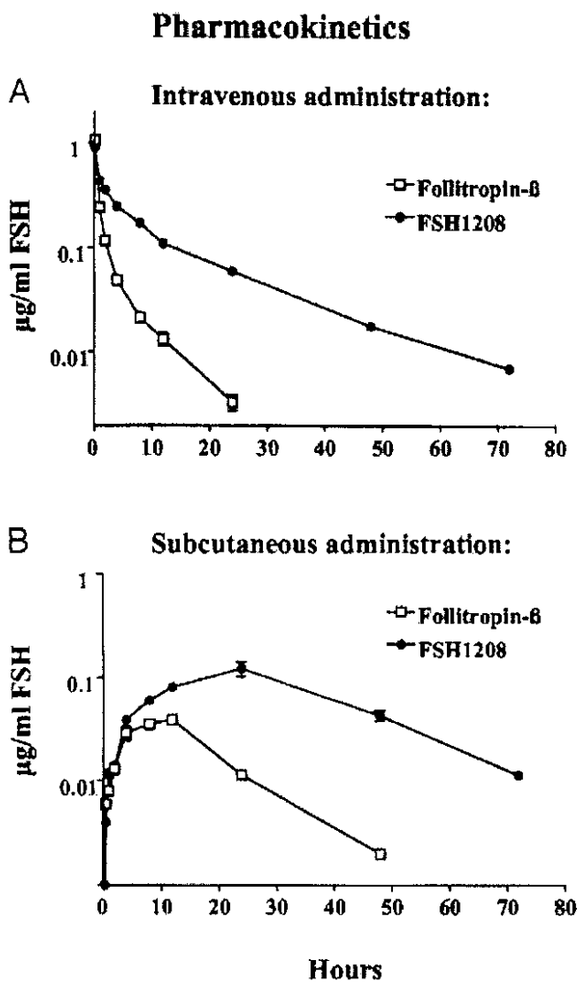


FIG. 3. Pharmacokinetic profile of an FSH $\alpha$-extension variant with two additional glycosylation sites (FSH1208) (●) and wild-type human recombinant FSH, follitropin-$\beta$ (□) in rats. A single dose of 10 $\mu$g FSH was administrated either iv (A) or sc (B).

was equal to the half-life measured after iv administration, *i.e.* 6 h, whereas FSH1208 had a half-life of 18 h after sc administration. The area under the concentration curve was 4 to 6 times larger for FSH1208 than for wild-type rFSH (follitropin-$\beta$), depending of the route of administration (Table 1). The studies indicate that the bioavailability of FSH1208 was about 80% and, thus, higher than of follitropin-$\beta$ of about 50%. *In vitro* bioactivity measurement showed that there was still FSH activity in the 48-h samples (data not shown).

*Pharmacodynamic studies*

The *in vivo* potency of the FSH forms was tested by analysis of their ability to augment ovarian weight in rats (24) on 48 or 72 h of stimulation. Furthermore, the estradiol level was measured in serum over a 48-h period. To test whether the increased half-life of FSH1208 translated into higher *in vivo* activity, FSH1208 was dosed only once, but follitropin-$\beta$ was dosed every day.

**TABLE 1.** Pharmacokinetics comparison of routes of administration for FSH1208 and wild-type rFSH, follitropin-$\beta$ with three animals in each group

| Parameter | FSH1208 | Follitropin-$\beta$ | *P* |
|---|---|---|---|
| AUC iv ($\mu$g/min/ml) | 309 ± 43 | 79 ± 3 | <0.001 |
| AUC sc ($\mu$g/min/ml) | 263 ± 82 | 42 ± 6 | <0.01 |
| Tmax sc (min) | 1440 ± 0 | 640 ± 139 | <0.01 |
| Cmax sc ($\mu$g/ml) | 0.13 ± 0.07 | 0.04 ± 0.004 | ns |

Mean ± SD. AUC, Area under the curve; Tmax, time of maximal concentration; Cmax, maximal concentration; ns, nonsignificant.

*Ovarian weight augmentation experiments*

The effect of FSH1208 during 48 h of stimulation was studied in an experiment in which all animals received a daily dosage of hCG and FSH or vehicle. Two doses of FSH1208 were studied: One group of animals was given 0.25 $\mu$g (approximately 0.6 IU), *i.e.* equal to the amount of follitropin-$\beta$ given to the rFSH group, and the other group was given 2.5 IU, equal to the amount of *in vitro* bioactivity given to the follitropin-$\beta$ group. Blood samples were drawn every 12 h for estradiol analysis, and the weight of the ovaries was measured after 48 h. In another experiment, the animals were stimulated for 72 h before they were euthanized, and the ovaries were decapsulated and weighed. The groups and the dosages were the same as in the previous experiment except the rats were treated for an extra day with either follitropin-$\beta$ or vehicle.

The ovarian weights after 48 h of stimulation are shown in Fig. 4A. All groups stimulated with either of the FSH formulations were significantly different from the control group stimulated with hCG only ($P < 0.001$–$0.01$). The group that received 2.5 IU of FSH1208 had a mean ovary weight on 51.8 ± 3.7 mg, compared with 34.9 ± 4.7 mg in the group that received follitropin-$\beta$ once ($P < 0.001$). In the 72-h stimulation experiment, the effect of FSH1208 was even more pronounced (Fig. 4B). Any dose of FSH1208, *e.g.* equal amount of protein or bioactivity, respectively, was markedly more potent than 1× follitropin-$\beta$. Interestingly, 0.25 $\mu$g FSH1208 given once was at least as efficient in augmenting the ovary weight as 0.25 $\mu$g follitropin-$\beta$ given once daily for 3 d, despite the fact that the specific activity for FSH1208 is only about one fourth of the specific activity of follitropin-$\beta$.

*Estradiol measurements*

Blood samples were drawn during the 48-h stimulation assay for analysis of the estradiol level in serum. Figure 5 shows the ELISA measurements of the estradiol level as a function of time. The curves suggest a circadian variation in the estradiol concentration after FSH stimulation, as previously described (34). It was not possible to detect any increase in the estradiol level during the first 12 h. After 24 h, a significantly higher content of estradiol ($P < 0.05$) was detected in the serum of the rats that were stimulated with either a dose of FSH1208, compared with the control group, or FSH1208 dosed in equal *in vitro* bioactivity (u), compared with 1× follitropin-$\beta$. The peak values of estradiol were measured after 24 h for all the groups with levels at a range from about 20 pg/ml for the control group to about 200 pg/ml for the FSH1208(u) group (Fig. 5).

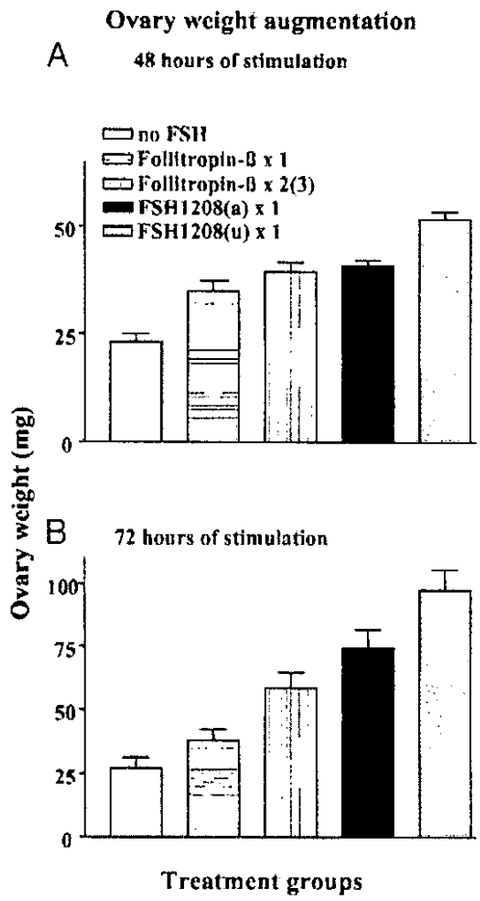


FIG. 4. Ovary weight augmentation after stimulation with FSH1208 and wild-type human recombinant FSH, follitropin-$\beta$, for either 48 h (A) or 72 h (B). A minimum of four rats in each group were stimulated with a daily dosage of hCG and FSH or vehicle. Rats in group 1 received only hCG (no FSH), rats in group 2 (G2) received 0.25 $\mu$g follitropin-$\beta$ once (follitropin-$\beta$ × 1), rats in group 3 (G3) received 0.25 $\mu$g follitropin-$\beta$ daily [follitropin-$\beta$ × 2 (A) or 3 (B)], rats in group 4 (G4) received 0.25 $\mu$g FSH1208 once [FSH1208 × 1 (amount)], and rats in group 5 (G5) received 2.5 IU FSH1208 once [FSH1208 × 1 (units)]. All groups stimulated with FSH were significantly different from the control group after both 48 and 72 h, except 1 × follitropin-$\beta$ after 72 h. CG *vs.* G2: $P < 0.01$ (48 h); CG *vs.* G3, G4, G5, respectively, after both 48 and 72 h: $P < 0.001$. 48 h: G2 *vs.* G5 ($P < 0.001$), G3 *vs.* G5 ($P < 0.01$), G4 *vs.* G5 ($P < 0.05$). 72 h: G2 *vs.* G4 and G5, respectively ($P < 0.001$); G3 *vs.* G5 ($P < 0.001$); G4 *vs.* G5 ($P < 0.05$).

The correlation between stimulation with gonadotropins and the follicular response is well described (35). The reported increase in number as well as in size of both antral and preovulatory follicle (36, 37) is a likely explanation for the enlargement of the ovaries and the increased peripheral estrogen levels seen in our study.

## Discussion

Introduction of additional glycosylation sites has successfully been used in the present study to produce a follicle-stimulating protein with prolonged serum half-life and increased *in vivo* activity. Strikingly, one dose of 0.25 $\mu$g of FSH1208, equivalent to about 0.6 IU, stimulates ovarian

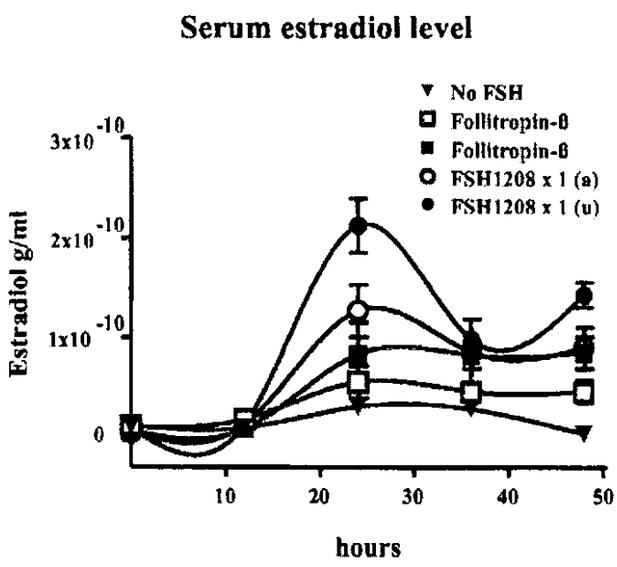


FIG. 5. 17$\beta$-Estradiol level in serum samples was measured over a 48-h period using ELISA. The rats were stimulated daily with hCG and FSH or vehicle. Group 1, received only vehicle (no FSH); group 2, 0.25 $\mu$g follitropin-$\beta$ once; group 3, 0.25 $\mu$g follitropin-$\beta$ both days; group 4, 0.25 $\mu$g FSH1208 once; group 5, 2.5 IU FSH1208 once. The FSH1208 groups were statically different from both the control group and the group that received 1 × follitropin-$\beta$ ($P < 0.05$).

growth and estradiol production at least as potently as daily dosages of the same amount of recombinant wild-type FSH, follitropin-$\beta$ (0.25 $\mu$g ~ 2.5 IU) for 2–3 d. FSH1208 was consistently more potent in rats than follitropin-$\beta$ when equal amounts of *in vitro* bioactivity were injected. This demonstrates that the increased *in vivo* half-life translates to a more potent *in vivo* action. In this study it has been shown that the prolonged half-life of FSH1208 is of major importance for increasing the *in vivo* activity. Recent studies suggest that apart from the metabolic clearance rate/terminal half-life, the binding affinity for the receptor and the efficiency in signal transduction also contribute to the net *in vivo* activity of a glycovariant (38, 39). The pharmacokinetic profile of FSH1208 results in a relatively stable serum concentration, which appears to be at least as effective as the high peaks and troughs obtained through daily injections of a molecule with a relatively short *in vivo* half-life such as rFSH, follitropin-$\beta$. This is in agreement with previous findings showing that several injections of small amounts of wild-type FSH, giving rise to a relatively constant concentration level, is more efficient than the total dose given as a single bolus injection (14).

In principle, endogenous rat FSH may have influenced the serum concentration measurements. However, if any such interference occurred, its effect on the results was only minor. Endogenous FSH levels in rats have been found to reach a maximum of about 10 ng/ml during the estrous cycle (40). In our experiments, the measured FSH levels are initially well below 10 ng/ml (0.01 $\mu$g/ml) (Fig. 3B) and drop well below this level at the last time points (Fig. 3, A and B). This indicates that the endogenous FSH levels must have been lower than 0.01 $\mu$g/ml or that our ELISA does not efficiently

detect rat FSH. In any event, even if the endogenous rat FSH levels would have been maximal throughout our experiment, the concentration curves lie for the most part well above the 0.01 μg/ml level. Furthermore, it is likely that the FSH treatment leads to reduction in endogenous FSH levels through pituitary feedback inhibition.

N-linked glycosylation has previously been used to alter the half-life of erythropoietin (EPO). EPO is a glycoprotein with three naturally occurring glycosylation sites and a molecular mass of approximately 30 kDa, thus fairly similar to FSH. A variant of EPO with two additional glycosylation sites (darbepoetin-α) was recently approved for the treatment of anemia. The molecular mass of darbepoetin-α is 8 kDa higher than EPO, and the *in vivo* terminal half-life was increased approximately 3-fold in rats, compared with EPO (41). These results are, thus, similar to what was observed in the present study. The terminal half-life in humans of darbepoetin-α was increased to the same extent, approximately 3-fold, and darbepoetin-α was able to stimulate erythropoiesis with less frequent dosing as effectively as recombinant human EPO (42, 43). The molecular size and the clearance mechanisms of FSH and EPO are very similar, and it seems reasonable to predict that the 3-fold increase in terminal half-life in rats for FSH1208 would be reflected in humans as well, just as it was observed for darbepoetin-α. FSH1208 may, thus, form the basis for an improved treatment of infertility with a reduced frequency of injections. This will naturally need to be verified in clinical studies.

Additional carbohydrate moieties may reduce the elimination of proteins because of the increase in size and charge. Sialic acid bears a net negative charge at physiological pH and makes a glycoprotein hormone more metabolically stable by decreasing the glomerular filtration (44, 45) and through protection against clearance by hepatic asialoglycoprotein receptors (46, 47). Microheterogeneity in the protein structure is seen for all glycoproteins and is caused by variation in the posttranslational glycosylation process in terms of differences in the degree of sialylation and different branching of the carbohydrate moieties. For FSH this gives rise to about 20 naturally occurring isoforms with different pI, biological activity and terminal serum half-life (48). The degree of sialylation of FSH changes both during the menstrual cycle and through the various stages of life. Acidic isoforms are mainly present in prepubertal and postmenopausal life and during the follicular phase of the cycle, whereas the majority of the isoforms are basic during puberty and during the midcyclic surge of FSH (49, 50). These changes in the secreted hormone from the pituitary gland are influenced by the endocrine milieu surrounding the gland and the current need/stage in the body (51–55). The differences in the degree of sialylation and metabolic stability of the different isoforms are reflected in the time the isoforms stay in circulation and, thus, the time they have to exert their effect. Sialic acid is, thus, of major importance in determining the half-life and, thus, the *in vivo* activity of a glycoprotein. Thus, a likely explanation for the changes in the *in vivo* characteristics of FSH1208, compared with recombinant wild-type FSH, follitropin-β, is a higher content of sialic acid, rather than just the amino acid extension or the presence of the remaining carbohydrate structures.

In the present study, two different approaches were used for introduction of new glycosylation sites: structure-aided, site-directed introduction of sites within the FSH molecule and addition of N-terminal extensions. A total of six sites identified within the structure were tested, and none of these were glycosylated to a large extent. All the changes in the native FSH molecule were made on the assumptions of the tertiary structure based on computer models and structural analysis. The single-point mutations were meant to be introduced on the surface of the molecule at sites believed to be unimportant for receptor and interchain interaction. The results suggest that the influence of the microenvironment on the glycosylation process is fairly unpredictable. The fact that only a few mutations were introduced and only at sites in which a threonine or serine already was located at position +2 together with the fact that the glycosylation process was not optimized by exchanging residues at important positions surrounding the glycosylation site (56, 57) minimized *a priori* the chances of identifying highly glycosylated muteins by simple point mutations. In contrast, most of the extension muteins were glycosylated and in the case of FSH1208 to a very high extent.

It has previously been shown that it is possible to make an extension of FSH without loosing the activity. A chimeric FSH-hCG molecule was created by fusion of the FSH β-chain with a sequence from the C terminus of hCG encompassing four possible O-linked glycosylation sites. The C terminus of the hCG molecule is responsible for the longer circulatory half-life of hCG, compared with the other glycoprotein hormones in the family and deletion of that region in hCG, has been shown to decrease the *in vivo* activity (58). The changed pharmacokinetic and dynamic profile of the chimeric FSH-hCG molecule are, thus, explained by the properties of the C terminus of hCG and probably by the sialic acids coupled to the four O-linked glycan structures. This chimeric molecule has recently been tested in clinical trials. The half-life in humans was prolonged and FSH-hCG induced multiple follicular growth in a dose-dependent manner. Furthermore, no safety problems were encountered (15, 59). The N-terminally N-glycosylated variant FSH1208 described here resembles the FSH-hCG chimera in the animal studies. However, because the two molecules have important structural differences, they may turn out to differ in clinical efficacy and/or appropriateness for large-scale manufacturing.

A consensus sequence is known for N-linked glycosylation (Asn-X-Thr or Asn-X-Ser), whereas no such clear motif is known for O-linked glycosylation (60–62). Thus, even though this study showed that N-linked glycosylation sites are not always used, the availability of the consensus sequence allows, with a reasonable change of success, to tailor-make protein extensions with any desired number of additional N-linked glycosylations. Thus, this approach is generally applicable and attractive for many other proteins (patent application WO 02/02597).

The change of the structure of FSH by introduction of a nonnative nine amino acid extension sequence might trigger an immune response in the body. However, the extension has a high extend of glycosylation, compared with the size of the extension itself ,and it is likely that a possible antigenic site in the amino acid core will be shielded by the carbohydrate

moieties (63, 64). Furthermore, it has been shown that the introduction of two additional glycosylated sites in darbepoetin-$\alpha$ does not cause an antibody response (42). It is possible that FSH1208 does not cause an immune response, but this remains to be demonstrated in clinical trials.

Differently charged forms of a glycoprotein have in addition to different half-lives also different biopotency. It is well established for many proteins, including FSH, that acidic isoforms are less potent *in vitro* than the isoforms with higher pI (10, 12, 23, 49, 65, 66). In line with this, FSH1208 with a lower pI profile has a reduced specific activity, compared with follitropin-$\beta$. The reduced specific activity of the charged glycoproteins is believed to be caused by reduced receptor-binding affinity, possibly because of charge-based repulsion and, thus, reduced signal transduction (67, 68).

In conclusion, introduction of additional glycosylation sites through addition of an N-terminal extension has been successfully used to create an FSH molecule with an increased *in vivo* half-life and prolonged *in vivo* activity. This molecule may form the basis for an improved treatment of infertility in the future.

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# Long-Acting Follicle-Stimulating Hormone Analogs Containing N-Linked Glycosylation Exhibited Increased Bioactivity Compared with O-Linked Analogs in Female Rats

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**The effects of altering the number and type of additional carbohydrate moieties on the pharmacokinetic and pharmacodynamic properties of FSH were examined in this report. A series of single-chain follitropins, containing variable numbers of additional N- (or O-) linked carbohydrates, were designed and expressed in Chinese hamster ovary cells. Proper folding, efficient receptor binding, and signal transduction were confirmed by *in vitro* assays. Pharmacokinetic and pharmacodynamic parameters were evaluated in immature female Sprague Dawley rats. Increasing the number of glycosylation sites with either N- (or O-) linked moieties extended the elimination half-life as much as 2-fold compared with recombinant human FSH (rhFSH). However, there was a maximum elimination half-life such that further glycosylation provided no additional lengthening of the half-life. Conversely, biopotency, as assessed by inhibin A levels 74 h post injection, and follicle production were significantly higher for the N-linked analogs. Rats stimulated with the longest acting analogs (either N- or O-linked) showed significantly higher ovarian weights than rats receiving a single injection of rhFSH. The analog containing four additional N-linked sites (rhFSH-N4) had the greatest number of large, preovulatory follicles. Although the half-life of rhFSH-N4 displayed no further enhancement beyond the other longest acting analogs, this analog exhibited significantly increased biopotency in rats. This work provides the basis for the generation of a series of reagents potentially useful for therapeutic applications. (*J Clin Endocrinol Metab* 89: 5204–5212, 2004)**

FSH IS A pituitary gonadotropin hormone that is important for testicular tubule development and ovarian follicle development. Exogenous FSH administration is an important component in assisted reproduction protocols to stimulate the development and maturation of oocytes (1–5). A disadvantage of FSH in this application is its relatively short half-life (6), requiring women to receive one to two im injections per day for 8–12 d in most protocols. Similarly, FSH replacement therapy in hypogonadotropic hypogonadal men requires three injections per week for several months. The development of a longer acting FSH analog with increased efficacy would reduce the number of injections and make such therapies more tolerable.

FSH, LH, TSH, and placental-derived human chorionic gonadotropin (hCG) are members of the glycoprotein hormone family. All family members are composed of two noncovalently bound subunits. The $\alpha$-subunit is shared by all family members, whereas the $\beta$-subunit is unique to each hormone and confers biological specificity (7). hCG has the longest circulating half-life of the members due to the presence of a unique carboxy-terminal peptide (CTP) on the $\beta$-subunit (8). Glycosylation of the CTP occurs at four O-linked carbohydrate attachment sites (9) and increases the terminal (elimination) half-life ($t_{1/2}$) of hCG by reducing glomerular filtration within the kidney (10–13). The CTP has also been shown to increase the half-life of recombinant human FSH (rhFSH) when covalently bound to the $\beta$-subunit (rhFSH-CTP) (14, 15).

rhFSH-CTP has recently been evaluated for human applications and was found to have a $t_{1/2}$ two to three times longer than native rhFSH in hypogonadotropic hypogonadal men after sc injection (16). Furthermore, in women, a single administration of rhFSH-CTP was sufficient to induce the growth of multiple follicles with a parallel rise in serum inhibin, a marker of follicular growth produced by granulosa cells (17, 18). The first live birth of a healthy infant was recently reported using an ovarian stimulation protocol incorporating rhFSH-CTP in combination with native rhFSH in a protocol requiring fewer injections than standard rhFSH-only regimens (19).

Efforts to further increase the *in vivo* bioactivity of FSH through the addition of two tandem CTP signal sequences showed no effect (14). The signal sequence for O-linked glycosylation is poorly defined, whereas N-linked glycosylation sites are specifically defined by the trimer peptide sequence

Abbreviations: CHO, Chinese hamster ovary; CTP, carboxy-terminal peptide; hCG, human chorionic gonadotropin; MALDI, matrix-assisted laser desorption ionization; pI, isoelectric point; rhFSH, recombinant human FSH; $t_{1/2}$, terminal half-life.

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Asn-X-Thr/Ser, where X can be any amino acid residue except proline. N-linked glycosylation sites can be introduced in tandem into FSH to generate a series of FSH analogs with variable, increased half-life and potentially enhanced *in vivo* bioactivity.

Previously, we reported the characterization and biological activity of a single- chain rhFSH construct, rhFSH-N2, that placed a synthetic polypeptide encoding two additional N-linked glycosylation sites between the β- and α-FSH subunits (20). The addition of the N-linked carbohydrate moieties did not interfere with proper folding of the hormone or binding to its receptor. rhFSH-N2 had similar *in vitro* activity to rhFSH and rhFSH-CTP and, like rhFSH-CTP, a $t_{1/2}$ that was 2-fold longer than rhFSH. Furthermore, other researchers have described increasing the half-life of rhFSH by introducing two N-linked glycosylation sites at the N terminus of the α-subunit (21). These results suggest that added N-glycosylation sites can increase half-life, regardless of the locations of the added sites within the hormone analog. Thus, novel FSH analogs containing variable numbers of N- (and O-) linked carbohydrates were examined in this report to determine how altering the number and type of additional carbohydrate moieties affects pharmacokinetic and pharmacodynamic properties of FSH. We found that increasing the number of glycosylation sites, either O-linked or N-linked, increased the $t_{1/2}$, but there was a maximum $t_{1/2}$ such that further glycosylation provided no additional benefit to prolongation of the half-life. However, when we compared the activities of these FSH analogs *in vivo* using several criteria that included comparisons of ovarian weights, inhibin A production, and follicle development, we found that N-linked analogs were significantly more biopotent than O-linked analogs.

## Materials and Methods

### *Construction of an rhFSH-N4 single-chain fusion clone*

We previously described rhFSH-N2, an expression vector that encodes the β- and α-subunits of FSH conjoined by a sequence encoding two N-linked carbohydrate attachment sites (20). All constructs described in this report were derived from rhFSH-N2. The N2 linker sequence between FSHβ and FSHα subunits was excised using the unique *Bam*H1 and *Spe*1 restriction sites present in the construct. New carbohydrate linker sequences, created through additional enzyme digestions or site-directed mutagenesis followed by PCR, were reintroduced into the original FSH plasmid by ligation. Fidelity of the new vector was confirmed by sequencing. Figure 1 shows the linker sequences for all other analogs described in this report.

### *Expression of rhFSH analogs in Chinese hamster ovary cells (CHO-K1)*

FSH constructs and $pSV_2neo$, an expression vector containing the selectable marker neomycin phosphotransferase, were cotransfected into CHO-K1 cells in the presence of Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA). G418-resistant colonies were selected in Ham's F12 medium containing 10% fetal calf serum (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, and 500 μg/ml G418 (Gemini Bioproducts, Woodland, CA) for 6–7 d and subsequently pooled together and maintained in the same medium. FSH production by pooled clones was detected by RIA using an antibody to β-FSH (Biodesign International, Saco, ME) as previously described (20). FSH-containing supernatants were obtained from cellular suspensions in spinner bottles that were initially seeded at $1 \times 10^5$ cells/ml into CHO-S-SFM II medium (Invitrogen) containing 400 μg/ml G418. Cells usually reached a density of $2 \times 10^6$ cells/ml on d 6 or 7, at which time cell supernatant was harvested on d 7 or 8. Supernatants were supplemented with 0.2 mM phenylmethylsulfonylfluoride, filtered through a 0.2-μm membrane, and kept at 4 C until purification.

### *Protein purification of rhFSH analogs from CHO-K1 supernatant*

rhFSH analogs were purified by adsorption and elution from an α-chain-specific monoclonal antibody column that was prepared by coupling 5 mg purified A103 Ig/ml to CNBr-Sepharose-4B (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions (22). After applying the cell supernatant, the column was washed in 50 bed volumes of PBS followed by 2 bed volumes of distilled water. The rhFSH analog was eluted in 3–4 bed volumes of 1 M acetic acid and immediately dried *en vacuo* in a Speed-Vac (Savant Instruments, Holbrook, NY).

### *Matrix-assisted laser desorption ionization (MALDI) analysis*

rhFSH analogs, prepared specifically for MALDI analyses, were purified by reverse-phase HPLC using a Hewlett-Packard 1090 (Hewlett-Packard, Palo Alto, CA) and a Vydac C4 column (Grace Vydac, Hesperia, CA). Twenty-five percent of peak fractions were dried *en vacuo* and

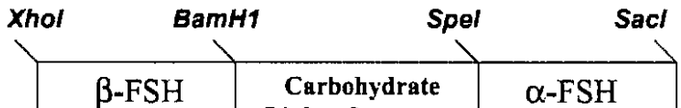


FIG. 1. Schematic of the rhFSH analog constructs. Diagram is not drawn to scale.

**Carbohydrate Linker Sequences:**

| | |
|---|---|
| rhFSH-N0: | GSGSGSGSGASGSGSGSGTS |
| rhFSH-O1: | GSPRFQDSSSSKAPPPRSTS |
| rhFSH-CTP: | GSPRFQDSSSSKAPPPSLPSPSRLPGPSDTPILPQTS |
| rhFSH-N1: | GSGSGSGSNATGSGSGSGTS |
| rhFSH-N2: | GSGSNATGSGSNATSGSTS |
| rhFSH-N4: | GSGSNATGSGSNATSGSGSNATGSGSNATSGSTS |

dissolved in 3 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in formic acid-propanol-water (1:2:3). A 0.5-μl aliquot was spotted on a sample plate prepared with a thin layer of α-cyano-4-hydroxycinnamic acid for mass spectrometric analyses in a PerSeptive Biosystems Voyager-DE RP MALDI mass spectrometer (PerSeptive Biosystems, Inc., Framingham MA).

### *Electrophoresis and Western blotting*

rhFSH (Gonal-F; Serono, Inc. Rockland, MA), single-chain rhFSH-CTP, rhFSH-N0, rhFSH-N1, rhFSH-N2, rhFSH-N4, and rhFSH-O1 were reduced and electrophoresed through an SDS-PAGE gel and silver stained (23). Another set of nonreduced samples were electrophoresed and transferred to nitrocellulose using standard techniques (23–25). The nitrocellulose was incubated overnight in a 1:10,000 dilution of mouse antihuman FSHβ monoclonal antibody (Biodesign International) after being blocked in 5% BSA for 1 h. The membrane was subsequently washed five times and incubated in 1:10,000 dilution of peroxidase-conjugated polyclonal antibody to mouse Ig (Amersham-Biosciences), for 1 h. Bands were visualized, after additional washes in Tris-buffered saline, by incubation in chemiluminescent detection reagent (Amersham-Biosciences) and exposed to x-ray film.

### *Isoelectric focusing gel electrophoresis*

Isoelectric points (pI) of FSH proteins (400 pmol/lane) were determined by electrophoresis through Novex pre-cast IEF gels containing a pI range of pH3–pH7 (Invitrogen, Carlsbad, CA). Samples were visualized by silver stain.

### *In vitro biology*

FSH concentration of all analogs was determined by RIA using a monoclonal antibody to FSHβ (Biodesign International). FSH receptor binding and activation *in vitro* was assessed by measuring cAMP activity in CHO cells expressing the hFSH receptor (CHO-FSHR) as previously described (26). CHO-FSHR cells were detached from culture dishes using 0.02% versene. Twenty thousand cells per tube were incubated with varying amounts of standard FSH or FSH analogs for 15 min at 37 C. Tubes were incubated for 3 min at 75 C and then centrifuged for 10 min at 750 × *g*. One hundred microliters of supernatant were assayed for cAMP concentration using a commercial RIA kit (Perkin-Elmer Life Sciences, Boston, MA).

### *In vivo bioactivity and half-life determinations*

Approval for animal experiments was obtained from the Institutional Animal Care and Use Committees at Serono (Rockland, MA) and Columbia University (New York, NY). Twenty-one-day-old female Sprague Dawley rats, obtained from Charles River Laboratories (Wilmington, MA), were housed three to five rats per cage and given standard food and tap water *ad libitum*. Animals were randomly assigned to receive a single injection of one of the following treatments either iv or ip: saline, rhFSH, rhFSH-N0, rhFSH-N1, rhFSH-N2, rhFSH-N4, rhFSH-O1, or rhFSH-CTP. There was no statistical difference in animal weights between each group. Each analog was injected at a dose of 42 IU (27–29), as determined by the FSH RIA.

Animals were anesthetized by inhalation of isofluorane before injection. Blood collection was obtained by periorbital venipuncture or tail bleeds. Rats were euthanized at the conclusion of the experiments by carbon dioxide inhalation followed by exsanguinations via cardiac puncture. FSH was detected in serum samples using RIA or Immulite (Diagnostic Products Corporation, Los Angeles, CA), and inhibin A levels were measured using an ELISA kit (Diagnostic Systems Laboratories, Webster, TX).

Pharmacokinetic analysis was performed using Winnonlin 1.0 software (Pharsight Corporation, Mountain View, CA) for iv injection and PK Solutions 2.0 software (Summit Research Services, Montrose, CO) for ip injections. For iv injections, mean values for each time point within a group were used to estimate pharmacokinetic parameters for $t_{1/2}$ and area under the serum concentration time curve. For ip injections, pharmacokinetic parameters were determined for each individual rat.

### *Histological analysis*

Rats were euthanized at 74 h post injection (ip), and ovaries were removed, weighed, fixed in paraformaldehyde, and embedded in paraffin. Each ovary was serial-sectioned completely at intervals of 10 μm. Mounted sections were stained in hematoxylin and eosin according to standard procedures. Ovaries were evaluated using a Leitz Dialux 20 light microscope equipped with a SPOT digital camera (Diagnostic Instrument, Inc., Sterling Heights, MI). Each section was evaluated for the presence of antral follicles. Follicles with clear antrum development, at stages 6–8 of the Pedersen and Peters grading system (30), were included in the follicle count. The ovary having the largest number of antral follicles within each treatment group was further evaluated for follicle size. Sizes of the first 30 (saline) or 50 (all other treatment groups) follicles with a clear antrum and the nucleus present in the section were measured using a Reichert eyepiece reticle, and these measurements were converted to actual follicle sizes per the manufacturer's instructions (Reichert Scientific Instruments, Buffalo, NY).

### *Statistical analysis*

Ovarian weights and inhibin levels were compared between groups in a one-way ANOVA model. The data were log transformed first to stabilize the variance between groups and satisfy the model assumptions because groups with higher means tended to have higher variances. The primary comparison of interest was between rhFSH-X (X = additional carbohydrate or tether) and rhFSH, and this difference was tested using a single contrast within the one-way structure. In addition, individual pharmacokinetic parameters (ip) were compared using a one-way ANOVA model. The comparisons of interest were each analog to FSH and additionally rhFSH-N4 *vs.* rhFSH-CTP and rhFSH-N4 *vs.* rhFSH-N2. These hypotheses were tested with simultaneous contrasts using a Bonferroni adjustment to control for multiple comparisons. Statistical significance of differences was assessed by ANOVA for one-way analysis or simultaneous comparison of FSH-N4 to all other groups using Dunnett's method (31). $P < 0.05$ was considered significant.

## Results

### *Biochemical analyses of the proteins*

rhFSH-CTP, rhFSH-N0, rhFSH-O1, rhFSH-N1, rhFSH-N2, and rhFSH-N4 were produced by CHO cells at concentrations of 1.3–14 pmol/ml in suspension culture. Western blot analysis showed that rhFSH migrated at an apparent mass of 41,000 Da (Fig. 2B). No additional carbohydrate was added to rhFSH-N0. The increased molecular mass (46,000 Da) compared with rhFSH was attributed to the additional 20 amino acids in the linker sequence (Fig. 2 and Table 1). The hyperglycosylated FSH analogs migrated more slowly than rhFSH-N0 (rhFSH-N1, 52,000 Da; rhFSH-CTP, 54,000 Da; hFSH-N2, 56,000 Da; and rhFSH-N4, 58,000 Da). The apparent molecular mass of rhFSH-O1 (44,000 Da), containing only a modest increase in carbohydrate, was similar to rhFSH-N0. Glycosylation can increase the sialic acid content of a protein, resulting in a more acidic profile that is detectable by a decrease in pI. Figure 3 shows that dimeric rhFSH exhibits a pI between 4.75 and 3.9 (lane 4), whereas the hyperglycosylated isoforms all exhibited a lower pI range of 4.45–3.5, supporting the assertion that rhFSH-CTP, rhFSH-N2, rhFSH-N1, and rhFSH-N4 are more glycosylated than rhFSH. The pI ranges for rhFSH-O1 (lane 8), rhFSH-N0 (lane 1), and rhFSH (lane 4) were similar probably because these proteins either lack or contain limited additional carbohydrate. The same amount of protein was applied to each lane, yet some analogs focused less discreetly (lanes 2, 7, and 8), which may be attributable to a greater number of isoforms for those analogs.

MALDI mass spectrometry is a quantitative method for analyzing molecular weight. It can be used to assess post-translational modifications that result in an increase of mass, such as by carbohydrate addition. The weight contributed by the carbohydrate component was estimated by subtracting the calculated weight of the polypeptide from the weight observed by mass spectrometry (Table 1). Noncovalently associated rhFSH $\alpha$- and $\beta$-chains, for example, exhibited two peaks that were measured at 12,034 and 16,569 Da, respectively, for a combined dimer mass of 28,603 Da. The peaks for single-chain rhFSH-CTP and rhFSH-N2 were larger than the rhFSH heterodimer, as expected (36,971 and 38,514 Da, respectively). rhFSH-N4, having four potential N-linked glycosylation sites, exhibited the largest molecular mass at 40,925 Da, which was a 30% increase over the rhFSH.

*In vitro bioactivity*

Increased glycosylation did not interfere with FSH activity because all FSH analogs stimulated the production of cAMP in CHO-K1 cells that constitutively expressed the FSH receptor (Fig. 4). rhFSH-N4, rhFSH-N1, rhFSH-O1, and rhFSH-N0 were able to stimulate a rise in cAMP levels comparable to rhFSH and other previously described analogs (rhFSH-N2 and rhFSH-CTP), indicating that the proteins were folded properly and were biologically active (Fig. 4).

*Pharmacokinetic studies*

Two sets of experiments were performed for half-life determination *in vivo*, wherein 22-d-old rats selected at random received single-dose injections of FSH analogs equivalent to 42 IU as assessed by RIA (27–29). In the first experiment, rats (n = 5 in each group) received a single dose of one of the following treatments through ip injection: saline, rhFSH, rhFSH-CTP, rhFSH-O1, rhFSH-N0, rhFSH-N1, rhFSH-N2, or rhFSH-N4. Blood was obtained by venipuncture via dorsal tail vein at the following intervals post injection: 3, 6, 9, 12, 24, 30, and 74 h. In the second experiment of a select group of analogs, rats (n = 3 in each group) received a single dose of one of the following treatments iv via dorsal tail vein: saline, rhFSH, rhFSH-CTP, rhFSH-N2, or rhFSH-N4. Blood was obtained by periorbital venipuncture at the following intervals post injection: 0.5, 1, 3, 6, 12, and 24 h. Rats were euthanized at 48 h post injection, and blood was collected by cardiac puncture. Serum FSH levels were measured as described in *Materials and Methods*.

Pharmacokinetic parameters for both iv and ip injection are depicted in Table 2. Additionally, mean concentration time curves for ip injection are illustrated in Fig. 5.

Table 2 shows that the increase in glycosylation can enhance the $t_{1/2}$ of the analogs. The $t_{1/2}$ values for all analogs containing additional carbohydrate were significantly higher

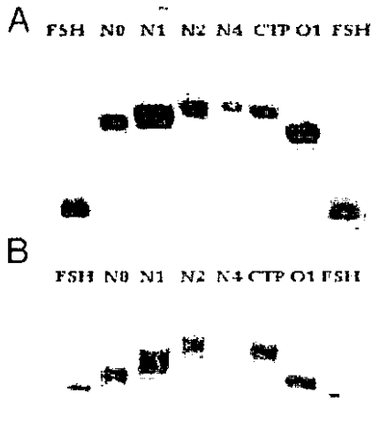


FIG. 2. Gel electrophoresis of the rhFSH analogs. The analogs have linker sequences containing variable amounts of additional carbohydrate (see *Materials and Methods*). Differences in molecular weight are a reflection of the changes in the carbohydrate content. A, Reduced silver-stained SDS-PAGE. Under reduced conditions, rhFSH dissociates into subunits that comigrate. B, Nonreduced Western blot visualized with an FSH$\beta$-specific antibody.

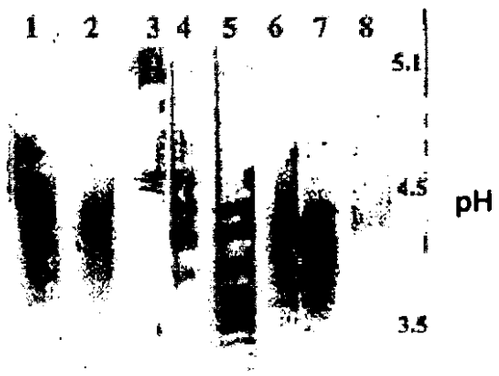


FIG. 3. Isoelectric focusing gel (pH 3–7) of the rhFSH analogs (lane 1, rhFSH-N0; lane 2, rhFSH-N1; lane 3, marker; lane 4, rhFSH; lane 5, rhFSH-N2; lane 6, rhFSH-N4; lane 7, rhFSH-CTP; lane 8, rhFSH-O1).

**TABLE 1.** Molecular weight of the rhFSH analogs

| Protein | Amino acids in tether (no.) | Calculated peptide molecular mass (Da) | Molecular mass by MALDI (Da) | No. and type of sugar groups | Calculated carbohydrate mass (Da) |
|---|---|---|---|---|---|
| RhFSH | 0 | 22,691 | 28,603 | 4N | 5,912 |
| RhFSH-N0 | 20 | 24,143 | 30,935 | 4N | 6,792 |
| RhFSH-O1 | 19 | 24,731 | 33,468 | 4N, 1O | 8,737 |
| RhFSH-CTP | 31 | 26,429 | 36,971 | 4N, 4O | 10,542 |
| RhFSH-N1 | 20 | 24,213 | 36,000 | 5N | 11,787 |
| RhFSH-N2 | 19 | 24,241 | 38,514 | 6N | 14,273 |
| RhFSH-N4 | 32 | 25,478 | 40,925 | 8N | 15,447 |

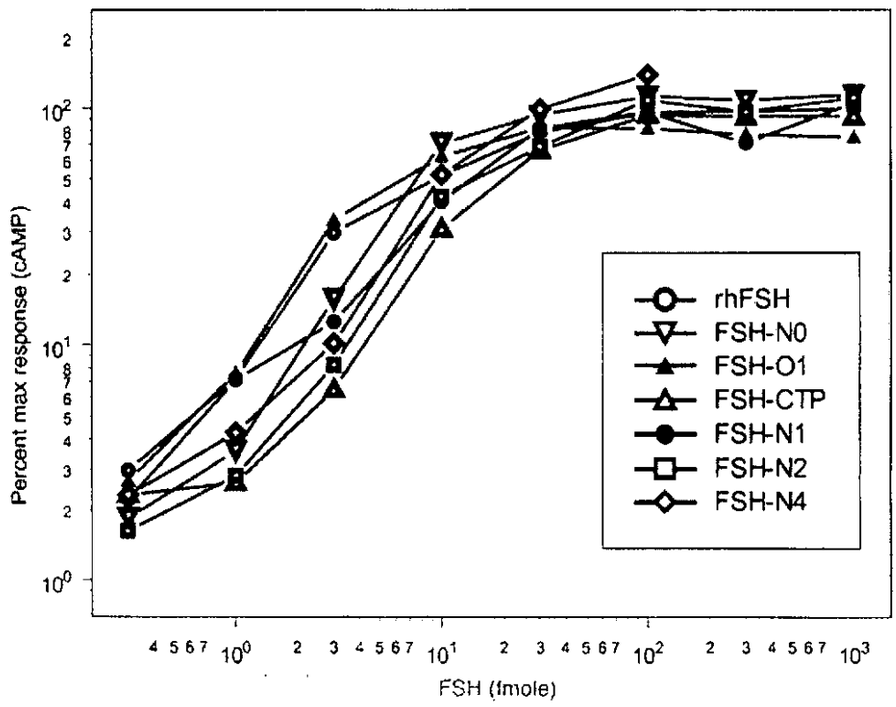


FIG. 4. *In vitro* bioactivity of rhFSH analogs. Adenylate cyclase activation by the different FSH analogs was determined using CHO cells expressing the FSH receptor. Stimulation of cells was conducted at 37 C for 15 min, and total cAMP was measured by RIA.

**TABLE 2.** Mean pharmacokinetic parameter estimates

| | FSH | N0 | O1 | CTP | N1 | N2 | N4 |
|---|---|---|---|---|---|---|---|
| Injection (ip) | | | | | | | |
| AUC (mIU-h/ml) | 12,172 | 16,832 | 11,776 | 36,102 | 35,995 | 52,656 | 58,419 |
| $t_{1/2}$ (elimination) (h) | 6.3 | 7.9 | 7.7 | 12.1 | 9.5 | 12.3 | 13.7 |
| Clearance (ml/h) | 3.51 | 2.66 | 3.70 | 1.22 | 1.19 | 0.81 | 0.74 |
| Injection (iv) | | | | | | | |
| AUC (mIU-h/ml) | 11,030 | | | 31,492 | | 32,032 | 26,719 |
| $t_{1/2}$ (elimination) (h) | 3.9 | | | 6.9 | | 7.9 | 7.8 |
| Clearance (ml/h) | 1.8 | | | 0.64 | | 0.63 | 0.75 |

AUC, Area under the concentration time curve.

than rhFSH. However, the modest increase in $t_{1/2}$ (ip) of rhFSH-O1 may be, in part, due to the linking of the subunits, given that the $t_{1/2}$ (ip) of rhFSH-N0 was increased by a similar amount. The $t_{1/2}$ (ip) of rhFSH-N1 was approximately 1.5 times higher than rhFSH. Although the $t_{1/2}$ (iv/ip) of rhFSH-N4 was approximately 2-fold longer than the $t_{1/2}$ of rhFSH ($P < 0.001$ for ip), it was similar to that of rhFSH-CTP and rhFSH-N2. Differences between $t_{1/2}$ (ip) values for rhFSH-CTP, rhFSH-N2, and rhFSH-N4 were insignificant ($P > 0.05$). Thus, the addition of carbohydrate prolongs the half-life of FSH with either N-linked and O-linked analogs, but increasing the number of glycosylation sites beyond N2 appears not to be correlated with a longer $t_{1/2}$.

*Pharmacodynamic studies*

Potency of each analog was evaluated *in vivo* using several parameters that included ovarian weight augmentation, ovarian follicle-specific hormone levels (inhibin A), and ovarian follicle development. All FSH therapies were administered as a one-time injection at dose equivalence of 42 IU (see *Materials and Methods*). No other hormones were given during the experiment.

*Ovarian weight augmentation*

Figure 6 shows mean ovarian weights at 74 h post treatment. Groups stimulated by rhFSH-CTP, rhFSH-N2, or rhFSH-N4 had significantly higher ovarian weights compared with saline or rhFSH ($P < 0.05$). rhFSH-N0 and rhFSH-O1 were similar to rhFSH, whereas rhFSH-N1 showed a slightly increased weight. rhFSH-N4 stimulated a higher mean ovarian weight of 22.5 mg compared with all other treatments, but it was not statistically more effective than rhFSH-CTP ($P > 0.05$) or rhFSH-N2 ($P > 0.05$).

*Inhibin A measurements*

Secondary preantral follicles produce inhibin A in rats, and an increase of inhibin A levels correlates with an increase in follicular growth (32). We found that inhibin A levels for rhFSH- and saline-treated control animals were

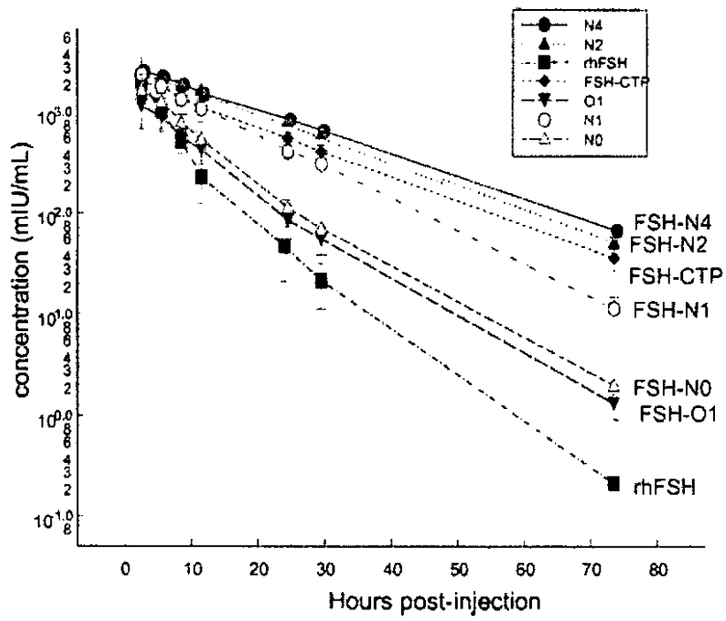


FIG. 5. Serum FSH concentration time profiles (ip). Immature female rats were injected with a single dose of 42 IU of each hormone analog. Serial bleeds were performed, and serum FSH concentrations were measured by Immulite (see *Materials and Methods*).

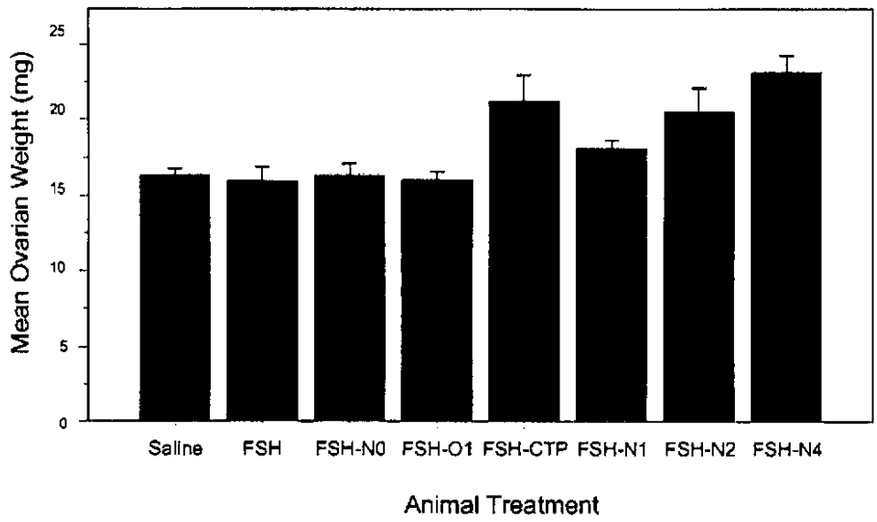


FIG. 6. Ovarian weights for analogs at 74 h. Ovarian weight augmentation after stimulation with FSH analogs. Immature female rats were injected with a single dose of 42 IU ip of each hormone analog and ovaries were removed, trimmed, and weighed 74 h post injection. Each treatment group contained a minimum of five rats.

similar at 74 h (Fig. 7), presumably due to the short $t_{1/2}$ of rhFSH at 6.3 h post ip injection (Table 2). To confirm that rhFSH was properly administered and biologically active, we measured inhibin A levels at 24 h and found that the inhibin A level was 60 times higher than at 74 h (data not shown). Hyperglycosylated forms of rhFSH induced inhibin A levels at 74 h that were significantly higher than the saline controls or rhFSH (Dunnet's method, $P < 0.05$; Fig. 7). A comparison of the O-linked analogs showed that CTP, for example, was able to induce significantly higher inhibin levels in the rats compared with rhFSH-O1, which is consistent with the theory that additional glycosylation leads to increased biological activity. All N-linked analogs stimulated significantly higher inhibin levels compared with the O-linked rhFSH-CTP. Even rhFSH-N1 induced more than twice as high inhibin A levels than rhFSH-CTP, suggesting that FSH activity is more efficient when the hormone is modified by N-linked glycosylation. The mean inhibin A level in the rhFSH-N4-treated group was the highest among all treatment groups at 6369 pg/ml and was statistically equivalent to rhFSH-N2 ($P = 0.3$). The O-linked glycosylated analog, rhFSH-CTP, was much less efficient at inducing inhibin A (1638 pg/ml) compared with rhFSH-N4 ($P < 0.001$). FSH analogs that contained only one N-link or one O-link glycosylation site showed the same differential characteristic where inhibin A levels in rhFSH-N1-treated animals (at 4580 pg/ml) were 40 times higher than rhFSH-O1-treated animals (Fig. 7). We conclude from these data that N-linked glycosylated analogs stimulate a larger number of active follicles than their O-linked structural counterparts.

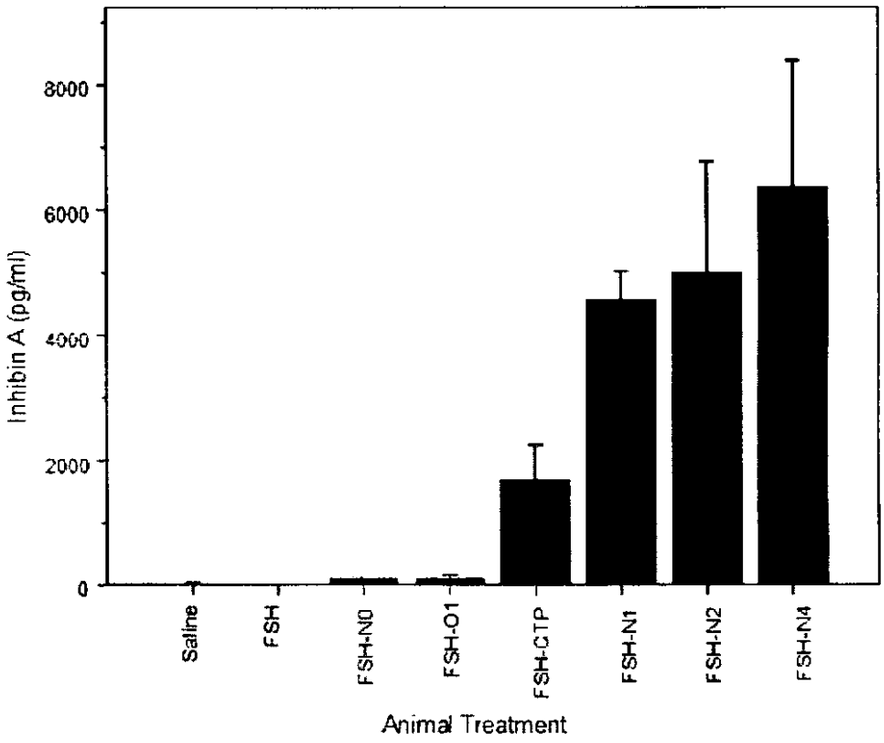


FIG. 7. Inhibin A levels for analogs at 74 h. Inhibin A levels after stimulation with FSH analogs. Immature female rats were injected with a single dose of 42 IU ip of each hormone analog. Inhibin A serum levels were assayed by ELISA 74 h post injection. Each treatment group contained a minimum of five rats. All N-linked analogs stimulated significantly higher inhibin levels compared with O-linked rhFSH-CTP.

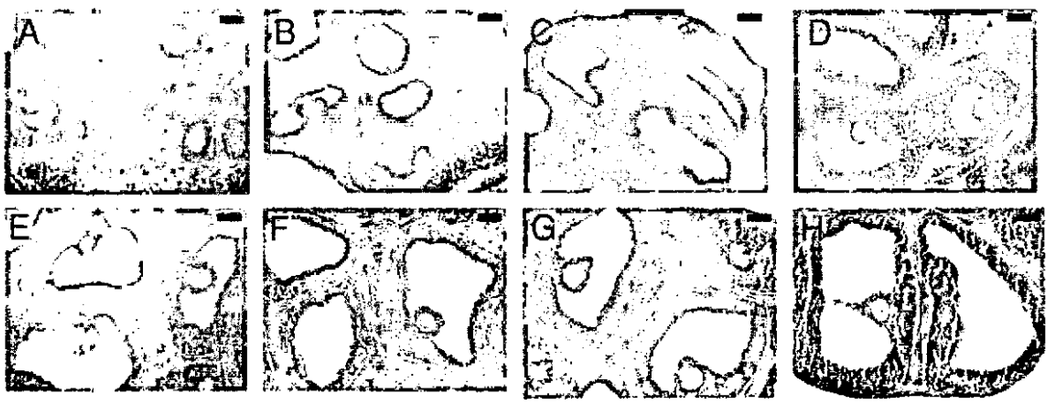


FIG. 8. Hematoxylin and eosin staining of rat ovaries 74 h post treatment. Magnification for all pictures, ×10. *Bar* equals 100 μm. A, Saline; B, rhFSH; C, rhFSH-N0; D, rhFSH-O1; E, rhFSH-CTP; F, rhFSH-N1; G, rhFSH-N2; and H, rhFSH-N4.

*Ovarian follicle development*

Antral follicle number and size was determined by examining hematoxylin and eosin-stained serial sections of ovaries from each treatment group (n = 4 ovaries in each group). Representative sections from each group at 74 h of exposure to analog are shown in Fig. 8. The saline-treated ovary shows a wide range of follicles from preantral to small, early antral follicles, whereas there were more antral follicles in all hormone-injected rats. Table 3 shows that the mean antral follicle count increased in all hormone-treated animals. rhFSH-N1 had 25% more antral follicles than rhFSH-CTP, but follicle size was similar for both analogs. rhFSH-N2- and rhFSH-N4-treated animals had twice as many antral follicles, and the mean size of the antral follicles were nearly 50% larger than rhFSH-treated animals (simultaneous $P < 0.0001$ using Dunnett's method). rhFSH-N4-treated animals produced the highest number of large, preovulatory antral follicles, which was determined by scoring the percentage of follicles ≥ 600 μm (saline, 0%; rhFSH, 0%; rhFSH-O1, 0%; rhFSH-CTP, 4%; rhFSH-N1, 2%; rhFSH-N2, 4%; and rhFSH-N4, 14%). The one-sided contrast showed that rhFSH-N4 did promote more follicle growth than rhFSH-N2.

## Discussion

Efforts to increase the half-life and biopotency of the pituitary gonadotropin FSH have focused on adding carbohydrate side chains to a linker fused to the native peptide backbone. The CTP of hCG contains four O-linked glyco-

**TABLE 3.** Ovarian follicle development at 74 h

| Treatment | No. of antral follicles per ovary | Mean size of antral follicles (μm) |
|---|---|---|
| Saline | 41 | 328 |
| rhFSH | 80 | 312 |
| rhFSH-N0 | 107 | 409 |
| rhFSH-O1 | 100 | 388 |
| rhFSH-CTP | 103 | 413 |
| rhFSH-N1 | 126 | 407 |
| rhFSH-N2 | 148 | 430 |
| rhFSH-N4 | 156 | 465 |

sylation sites and was the first hyperglycosylated moiety demonstrated to significantly increase the half-life and biopotency of FSH when attached to the C terminus of the β-subunit (14). Previously, we reported that the $t_{1/2}$ and biopotency could be enhanced through the addition of N-linked glycosylation sites as well (18). We and others had suggested that follitropin analogs that have different pharmacokinetic profiles and biopotencies *in vivo* could be produced by altering the number and types of carbohydrate moieties added post translationally (33). We tested this hypothesis here by examining the pharmacokinetics and bioactivity of rhFSH analogs that contained linker sequences encoding zero to four additional N-linked glycosylation sites and one to four additional O-linked glycosylation sites.

We found that the addition of as many as four N-linked carbohydrate moieties to rhFSH did not interfere with protein folding or *in vitro* activation of the FSH receptor. Thus, it is possible to add multiple carbohydrate chains and mass to FSH without adversely affecting its biological activity. The upper limit of total carbohydrate mass that is permissive or conducive to hormone activity remains unknown.

Hyperglycosylation increased the half-life of FSH, but its effect was not linear with respect to carbohydrate size and number. rhFSH-N4 and two other (smaller) previously described hyperglycosylated proteins, rhFSH-CTP (14, 15) and rhFSH-N2 (20), had similar $t_{1/2}$ kinetics. Thus, there does appear to be a maximum increase in half-life that can be achieved with glycosylation alone.

Previous pharmacology studies have shown that FSH is excreted by the kidney and that acidic endogenous isoforms of FSH are cleared more slowly than less acidic forms, regardless of carbohydrate type or amount (11–13). Our analysis shows that glycosylation lowers the pI profile and is most likely attributable to an increase in the sialic acid content of the carbohydrate side chains. Sialylation is known to confer a longer $t_{1/2}$ upon native pituitary FSH isoforms *in vivo* (34), and our study showed that the half-lives of the more acidic analogs, rhFSH-CTP, rhFSH-N2, and rhFSH-N4, were twice as long as rhFSH. rhFSH-N0 and rhFSH-O1, in contrast, had pI profiles similar to rhFSH and half-lives only slightly longer than the native hormone. This increase may be attributable, in part, to the tether. The pI profile and the half-life of FSH-N1 were intermediate between rhFSH and the more heavily hyperglycosylated analogs. Furthermore, we have shown that rhFSH-N2 and rhFSH-N4, which are 5% and 9% larger than rhFSH-CTP, respectively, display similar half-lives (Table 1 and Fig. 2). Recently, it has been shown that additional glycosylation at the amino-terminal side of the α-subunit also increases the $t_{1/2}$, indicating that the position of the added carbohydrate does not influence the pharmacokinetics (21).

Two of these analogs, rhFSH-N2 and rhFSH-N4, induced more gonadotropic activity *in vivo* than rhFSH-CTP, which has four O-linked glycosylation sites. This effect may be due to several factors including monosaccharide composition and branching within the carbohydrate moieties, the nature of the glycosyl linkage, or because N-linked analogs have a higher carbohydrate mass than the O-linked rhFSH-CTP.

Prolonged $t_{1/2}$ alone of our FSH analogs cannot adequately explain differences of *in vivo* bioactivity. The $t_{1/2}$ of rhFSH-N1, for example, was shorter than rhFSH-CTP, but it induced 2.5 times more inhibin A (Fig. 7). Furthermore, rhFSH-N2 and rhFSH-N4 induced inhibin A levels three and four times higher than rhFSH-CTP, respectively, despite a negligible difference in $t_{1/2}$. Thus, N-linked sugars augment gonadotropic activity independent of alterations in pharmacokinetic properties in a manner that is disproportionate to O-linked counterparts. The mechanism that is responsible for this effect is not known.

rhFSH-N2 and rhFSH-N4 stimulated the same number of antral follicles (Table 3), but rhFSH-N4 stimulated higher inhibin A levels (Fig. 7) and a higher percentage of follicles that grew to more than 600 μm (see *Results*). rhFSH-N2 and rhFSH-N4 have similar half-lives, similar pI profiles, and differ in size by only 6% (according to MALDI analysis and gel electrophoresis), leading us to hypothesize that rhFSH-N4 is a more efficient stimulator of follicle growth, possibly due to glycosyl chain length or steric hindrance. rhFSH-N2, for example, has only two additional glycosylation sites and may experience less spatial encumbrance than rhFSH-N4, thereby facilitating the addition of large, highly branched carbohydrates at each location. Alternatively, rhFSH-N4 has more carbohydrate moieties, and these chains may have fewer bifurcations due to increased spatial obstruction. This interpretation is supported by a study that showed an inverse relationship between FSH bioactivity and carbohydrate complexity (11).

Two recent clinical trials have shown that hyperglycosylation increases the half-life of FSH (19, 20), where the $t_{1/2}$ of rhFSH-CTP was two to three times longer than rhFSH. An increase in inhibin B levels was detected in hypogonadotropic hypogonadal men injected with rhFSH-CTP, validating its effectiveness in this clinical population (16). We speculate that rhFSH-N4 will have enhanced bioactivity in men and women, enabling fewer injections with comparable or improved results. In particular, rhFSH-N4 might be adapted for stimulating optimal spermatogenesis in hypogonadotropic hypogonadal men who have typically required a prolonged therapy of follitropin injections in conjunction with hCG. These carbohydrate-rich FSH analogs may also have enhanced efficacy, as is illustrated by a case report of a woman who did not respond adequately to current rhFSH formulations but who was able to produce 10 large follicles after rhFSH-CTP administration, which culminated in the birth of a healthy infant (19).

Our studies have shown that FSH analogs containing multiple N-linked glycosylation sites, such as rhFSH-N2 and rhFSH-N4, are more biopotent than those with multiple O-

glycosylation sites. The addition of multiple carbohydrate side chains does not appear to interfere with protein folding, and in fact, amplified glycosylation further enhances ovarian follicle growth. Furthermore, N-linked analogs can be designed more easily because signal sequences are known for N-linked glycosylation. Such FSH agonists represent a novel and potentially more powerful group of therapeutic candidates. Additional studies are needed to evaluate aspects of carbohydrate structure in bioactivity modulation. These include different branching patterns of carbohydrates in our analogs, determination of the optimal spacing for these glycosylation sequences, and the influence of additional N-linked glycosylation sites on bioactivity.

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