OBJECTIVE: To understand the properties of each available gonadotropin preparation, especially in terms of the differences between urinary-derived and recombinant preparations.

STUDY DESIGN: Human menopausal gonadotropin (hMG), highly purified urinary-derived follicle-stimulating hormone (uFSH-HP) and recombinant FSH (rFSH) were subjected to 2-dimensional gel electrophoresis (2-DE), and protein spots were visualized by silver-staining procedures. Major spots were analyzed by mass spectrometry. Fluorescent-labeled preparations were also subjected to 2-DE to evaluate the quantities of FSH isohormones contained in each preparation.

RESULTS: 2-DE and mass spectrometry analyses of hMG identified many extracellular proteins as major impurities and several plasma membrane proteins including prion proteins. Both uFSH-HP and rFSH demonstrated slight impurities and showed several alpha and beta subunit isohormones. rFSH contained higher amounts of the basic isohormones of the a subunit than uFSH-HP, whereas the predominance of the basic isohormones was less significant in the beta subunit.

CONCLUSION: Proteomic analyses demonstrated the detailed protein profiles of each preparation. Differences in the quantities of a subunit isohormones may contribute to the variations in FSH activity observed between recombinant and urinary-derived FSH preparations. (J Reprod Med 2009;54:459–466)

Keywords: follicle-stimulating hormone, gonadotropin preparations, isohormone, proteomic analysis, prion protein.

We demonstrated that prion proteins can be detected in hMG preparations by MS, suggesting that there is potential risk for developing prion disease in using hMG preparations.

Postmenopausal human urinary extract preparations have been successfully used in the treatment of human infertility since the 1960s. The desirability of having a highly purified product led to the development of immunopurified urinary follicle-stimulating hormone (FSH) preparations, for example, highly purified urinary FSH (uFSH-HP). Using urinary-derived preparations leads to problems such as batch inconsistency, unreliable supply, unwanted effects resulting from impurities and risks from using biologic substances; thus recombinant FSH (rFSH) was introduced in the mid 1990s and is becoming a mainstream agent for controlling ovarian stimulation. However, many clinicians still prefer to use urinary-derived preparations primarily because they contain a luteinizing hormone (LH) component. Although the necessity of LH supplementation during follicle stimulation in in vitro fertilization (IVF) remains controversial, it has been reported that LH supplementation is beneficial to older woman and patients with high endogenous LH levels after gonadotropin-releasing hormone (GnRH) agonist down-regulation. To supply LH, clinicians have to use human menopausal gonadotropin (hMG) and/or human chorionic gonadotropin (hCG) extracted from pregnant women's urine because recombinant LH is not commonly available at present. Another reason for the use of urinary-derived preparations is that these preparations are generally less expensive than recombinant preparations. In a practical sense, the cost-effectiveness of gonadotropin preparations should be discussed based on the prices of locally available urinary products. A few studies have reported that the cost per pregnancy is lower for rFSH than for urinary gonadotropin. Many studies have been performed to compare the efficacy and clinical outcomes of urinary-derived and rFSH preparations; however, no clear conclusions have been reached, and some meta-analyses have demonstrated contradictory conclusions. Of interest, it was recently reported that uFSH-HP is more effective than rFSH in older women based on a controlled randomized study.
We identified nongonadotropin urinary protein contaminants in hMG preparations that may affect the properties of these preparations.

Considering the widespread use of rFSH and urinary-derived gonadotropin, it is important to understand the properties of each available preparation, especially in terms of the differences between urinary-derived and recombinant preparations in order to optimize ovarian stimulation. To our knowledge, few studies have attempted to detect impurities in gonadotropin preparations; one study demonstrated the presence of several nongonadotropin proteins using sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PGE) and Western blotting. In this study, we performed exhaustive analyses of popular FSH preparations using a proteomic approach to investigate the detailed properties of each preparation. We also compared the quantities of FSH alpha and beta subunit isoformes between urinary-derived and rFSH preparations.

**Materials and Methods**

**Materials**

hMG in the form of Humegon 75 IU (lot 8324116, Organon, the Netherlands) or hMG Fuji 150 IU (lot AB07A, Fujipharma, Tokyo, Japan), highly purified urinary-derived FSH (uFSH-HP): Fertinorm P (lot no, Y03A1820, Serono, Geneva, Switzerland) and rFSH in the form of Folistim (lot no, 80045455, Organon) were purchased and analyzed in this study. CyDyes, DryStrips, IPG Buffer, 2D gel (ExcelGel XL 12-14) and a silver stain kit (PlusOne) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, U.K.). Urea, thiourea, dithioerythritol (DTT), Tris, SDS, HCl, glycerol, acetonitrile and formic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). 3-[(3-Cholamidopropyl) dimethylammonio] 1-propanesulfonate (CHAPS) and iodoacetamide were purchased from Sigma (St. Louis, Missouri). Proteomics grade trypsin was bought from Roche (Basel, Switzerland).

**Sample Preparation**

Humegon, hMG Fuji, Fertinorm P and Follistim preparations were dissolved in lysis buffer (7.5 M urea, 2 M thiourea and 4% [w/v] CHAPS). Protein concentrations were determined by the Bradford method with bovine serum albumin (BSA) as the standard. Fifty micrograms of proteins were mixed with rehydration buffer (7.5 M urea, 2 M thiourea, 4% [w/v] CHAPS, 2% [w/v] DTT and 0.5% IPG buffer [pH 3–10]). The protein mixture (30–70 IU FSH, Fertinorm P and Follistim preparations, and 25 µg carrier protein [bovine XOR: MW 150 kD]) was labeled with 200 pmol of CyDye (minimal dyes), Cy3 and Cy5, respectively, according to the manufacturer’s protocol. The labeled samples were combined and mixed with the rehydration buffer described previously. The prepared samples were applied to a 24-cm DryStrip (pH 3–10) for overnight rehydration.

**2-DE**

The first dimension of isoelectric focusing (IEF) was conducted for 35,000 volt-hours (Vh) at 20°C using the Multiphor II system according to the operating manual of GE Healthcare. The first-dimension strips were first soaked in 10 mL of equilibration buffer A (50 mM Tris/HCl, pH 6.8 at 4°C, 30% [v/v] glycerol, 1% [w/v] DTT, 2% [w/v] SDS, and 6 M urea) and then in 10 mL buffer B (50 mM Tris/HCl, pH 6.8 at 4°C, 30% [v/v] glycerol, 2.5% iodoacetamide, 2% [w/v] SDS and 6 M urea). The strips were then subjected to electrophoresis on a second-dimension polyacrylamide gel (ExcelGel XL-SDS 12–14).

Silver-stained gels were scanned using an ES-2200 (Epson, Suwa, Nagano, Japan). CyDye-labeled protein spots were visualized using a Typhoon 9400 imager (GE Healthcare). The quantification of protein spots was carried out using Image Quant TL software (GE Healthcare). Three analytical replicates were performed for each sample to confirm the reproducibility of the protein spot pattern on 2-DE.

**Protein Identification by Mass Spectrometry**

The protein spots were excised and washed in ultrapure water, followed by acetonitrile, before being dried under a vacuum. The proteins in the gels were reduced with 100 mM dithiothreitol at 56°C for 30 minutes and alkylated with 100 mM iodoacetamide at 37°C for 30 minutes in the dark. The gels were then washed in acetonitrile and dried under a vacuum. The dried gels were reswollen by adding 10 mM Tris-HCl buffer (pH 8.8 at 37°C) containing 12.5 ng/µL trypsin. In-gel digestion was performed with 10 mM Tris-HCl buffer (pH 8.8 at 37°C) and incubation overnight at 37°C. After peptide extraction with the extraction buffer, 70% CAN, and 5% formic acid, the extracted peptide mixture was dried and dissolved with 20 µL of 0.1% trifluoroacetic acid (TFA). Mass spectrometry was performed at the Mass Spectrometry Facility (Department of Hygiene and Public Health, Nippon Medical School). Peptides were subjected to a high-pressure liquid

chromatography (HPLC) separation on a MAGIC 2002 (Michrom BioResources, Auburn, California). The peptides were separated onto a reversed-phase capillary HPLC column (C18, 200 angstrom, 0.2 × 50 mm, Michrom BioResources). As solvents, 2% acetonitrile in 0.1% formic acid (solvent A) and 90% acetonitrile in 0.1% formic acid (solvent B) were used with a linear gradient of 5–65% of solvent B over 50 minutes. The chromatography system was coupled via an HTS-PAL (CTC Analytics, Zwingen, Switzerland) to an ion trap mass spectrometer and an LCQ DECA XP ion trap mass spectrometer (Thermo Finnigan, San Jose, California). The resulting mass spectrometry (MS) and tandem mass spectrometry (MS/MS) spectra of peptide ions were searched for using Mascot at http://www.matrixscience.com.

Results

Protein Profiles of uhMG Preparations

2-DE analyses of the hMG preparation (Humegon) showed spots located between pH 4 and 8 and of molecular sizes between 10 and 75 kDa (Figure 1A). Liquid chromatography (LC)-MS/MS analyses identified many extracellular proteins, including MAC25/insulin-like growth factor binding protein 7, fibrillin-1, apoprotein D, EGF-containing extracellular matrix protein 1, alpha-1-microglobulin and neutrophil gelatinase-associated lipocalin as major impurities. Spots corresponding to the beta subunit of FSH were identified; however, the presence of isoforms of the gonadotropin alpha and beta subunits of LH could not be demonstrated. Focused analyses using a pH range of 3–5.6 resulted in additional detection of other impurities including prion proteins (Figure 1B). Three analytical replicates were performed to confirm the reproducibility of the prion protein detection. The results of the MS analyses of this hMG preparation are summarized in Table I. 2-DE analyses of the other hMG preparation, hMG Fuji 150, showed a different spot pattern, and several molecules were newly identified as major impurities (Figure 1C, Table II).

Protein Profiles of Urinary-Derived Immunopurified Human FSH and Recombinant Human FSH Preparations

Both immunopurified uFSH and rFSH demonstrated slight impurities and showed several isoformes of the FSH alpha and beta subunits, in the pH ranges 3–4 and 4–5, respectively (Figure 2A and B).

Table 1  The Protein Profile of the hMG Preparation (Humegon 75 IU, lot 8324116)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gi no.</th>
<th>MW</th>
<th>Pl</th>
<th>Spot no.</th>
<th>Function of protein</th>
<th>Localization of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate-binding</td>
<td>182422</td>
<td>26261</td>
<td>8.3</td>
<td>38</td>
<td>Receptor activity</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Ig gamma 1</td>
<td>226787</td>
<td>25229</td>
<td>7</td>
<td>40</td>
<td>Immune response</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Ribonuclease, RNaase family, 2</td>
<td>4506549</td>
<td>18354</td>
<td>9.14</td>
<td>42, 43, 44</td>
<td>Ribonuclease activity</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>4189275</td>
<td>66035</td>
<td>5.69</td>
<td>1</td>
<td>Transport/cargo protein</td>
<td>Extracellular</td>
</tr>
<tr>
<td>S100 calcium-binding protein A9</td>
<td>4206773</td>
<td>13241</td>
<td>5.71</td>
<td>5</td>
<td>Calcium ion binding</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>MMP9 (collagenase IV)</td>
<td>116863</td>
<td>78427</td>
<td>5.69</td>
<td>11</td>
<td>Protein metabolism</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>38051823</td>
<td>90584</td>
<td>6.89</td>
<td>23, 43, 44</td>
<td>Peptidase activity</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Fibroconnectin</td>
<td>31397</td>
<td>256689</td>
<td>5.45</td>
<td>30</td>
<td>Cell growth and/or maintenance</td>
<td>Extracellular</td>
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<tr>
<td>Peptidoglycan recognition protein 1</td>
<td>4827036</td>
<td>21730</td>
<td>8.92</td>
<td>33</td>
<td>Immune response</td>
<td>Unknown</td>
</tr>
<tr>
<td>Immunglobulin heavy chain, constant region</td>
<td>2414494</td>
<td>24489</td>
<td>6</td>
<td>40</td>
<td>Immune response</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Delta-like 1</td>
<td>110735443</td>
<td>70055</td>
<td>5.85</td>
<td>20</td>
<td>Calcium ion binding</td>
<td>Nucleus</td>
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<tr>
<td>Chain A, solution structure of hMIF 20202</td>
<td>157874502</td>
<td>17856</td>
<td>5.89</td>
<td>12</td>
<td>Signal transduction</td>
<td>Plasma membrane</td>
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<tr>
<td>Epidermal growth factor</td>
<td>4503491</td>
<td>133946</td>
<td>5.56</td>
<td>45, 46</td>
<td>Growth factor activity</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Prion protein</td>
<td>190470</td>
<td>26814</td>
<td>9.2</td>
<td>47</td>
<td>Metabolism</td>
<td>Plasma membrane</td>
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</table>

Table 2  The Protein Profile of the hMG Preparation (hMG Fiji 150 IU, lot AB07A)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gi no.</th>
<th>MW</th>
<th>Pl</th>
<th>Spot no.</th>
<th>Function of protein</th>
<th>Localization of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-acid glycoprotein 1 precursor</td>
<td>1197299</td>
<td>23579</td>
<td>5.01</td>
<td>6, 7, 8, 11, 12</td>
<td>Immune response</td>
<td>Extracellular</td>
</tr>
<tr>
<td>α1-antitrypsin</td>
<td>177831</td>
<td>22071</td>
<td>6.11</td>
<td>5, 10, 13, 14, 15, 16</td>
<td>Protease inhibitor activity</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>α2-plasmin inhibitor</td>
<td>219408</td>
<td>31195</td>
<td>5.39</td>
<td>17</td>
<td>Protease inhibitor activity</td>
<td>Extracellular</td>
</tr>
</tbody>
</table>

Protein Profiles of Urinary-Derived Immunopurified Human FSH and Recombinant Human FSH Preparations

Both immunopurified uFSH and rFSH demonstrated slight impurities and showed several isoformes of the FSH alpha and beta subunits, in the pH ranges 3–4 and 4–5, respectively (Figure 2A and B).

Figure 2  2-DE analysis of FSH preparations. Protein spots of isoformes corresponding to FSH α and β subunits are marked by circles. Both the pH values and molecular masses of the marker proteins are indicated. (A) uFSH-HP (Fertinorm P, lot Y03A1820). (B) rFSH-Follistim Injection 150 IU, lot 80045455. (C) Green spots: uFSH-HP (Fertinorm P, lot Y03A1820). Red spots: rFSH (Follistim injection 150 IU, lot 80045455). Yellow spots: overlapping area.
On 2-DE mapping of the mixture of different-colored fluorescent-labeled proteins, both uFSH-HP and rFSH demonstrated 6 isohormones of the FSH alpha subunit and 8 isohormones of the beta subunit at identical pH levels and molecular sizes (Figure 2C). A fluorescent-labeled carrier protein, bovine XOR, was also applied to 2-DE alone. The result confirmed that the protein spot is located at 150 kD, which is distant from the spot positions corresponding to FSH (data not shown).

**Quantitative Analysis of the FSH Isohormones Contained in Immunopurified uFSH and rFSH Preparations**

Based on the intensities of the fluorescent-labeled protein spots, rFSH contained higher amounts of the basic isohormones of the FSH alpha subunit than uFSH-HP (Figure 3A). In contrast, the differences in the levels of basic isohormones between rFSH and uFSH-HP were less significant for the FSH beta subunit (Figure 3B).

**Discussion**

In this study, we identified many proteins other than gonadotropin in hMG preparations. Most of the protein spots demonstrated by 2-DE were located at points different from their theoretical isoelectric point (pI) and original molecular weights, indicating that these proteins had been degraded and/or altered during the manufacturing process. Therefore these impurities do not necessarily affect the preparation properties because they may not be bioactive; however, altered or degenerated proteins can potentially be a source of immunogenic molecules. We failed to demonstrate the alpha subunit of gonadotropin or the beta subunit of LH (or hCG) during the 2-DE analyses of the hMG preparations. Both of these subunits should have been contained in the hMG preparations to exert FSH and LH activity; however, it might be difficult to detect them using this study system because a high extent of glycosylation can impair the detection sensitivity of mass fingerprinting analysis. 2-DE analyses of uFSH-HP and rFSH did not demonstrate the presence of nongonadotropin proteins. Although these results do not exclude the presence of minute amounts of impurities both preparations were highly purified in agreement with past reports that have shown that the purity of immunopurified urinary FSH is >95%15 and that of rFSH is >99.9%.16

FSH, like the other gonadotropins LH and hCG, exists in different molecular forms, which differ in their oligosaccharide structures, particularly in their degree of terminal sialylation.17,18 The degree of sialylation causes basic-acidic charge differences, which provide the basis for differences in receptor binding activity and metabolic clearance rate.17 Although the amino acid sequence of rFSH matches that of natural human FSH, differences in the quantities of FSH isohormones have been reported. Based on enzyme immunoassays that counted the chromatofocused fractions of preparations, it has been reported that rFSH contains about 2-fold more basic isohormones with a pI >4.7 and 2-fold less acidic isohormones with a pI <4.1 than uFSH-HP.19,20 Immunoassay provides a relative measure of the amounts of various molecules by recognizing particular structural features.21 In this study, we directly assessed the quantity of each alpha and beta subunit isohormone by analyzing the intensity of fluorescent-labeled spots. We demonstrated that the preponderance of relatively basic FSH isohormones in rFSH can be mainly attributed to the presence of alpha subunits. It was also indicated that the isohormones that constitute uFSH-HP and rFSH are the same because the locations of the protein spots were identical between the preparations on 2-DE maps. Recently, the crystal structure of human FSH in complex with its receptor was demonstrated; this suggested that the receptor binding of the hormone is mediated by key interaction sites involving both the alpha and beta subunits.22 Our results indicate that the differences in FSH activity between rFSH and uFSH-HP preparations are predominantly caused by differences in the quantities of a subunit isohormones.
Prions are naturally produced pathogenic forms of proteins, which are mainly formed by nerve cells. The normal and abnormal prion forms are referred to as prion protein cellular (PrPc) and prion protein scrapie (PrPSc), respectively. All types of prion disease are associated with the accumulation of PrPSc within the central nervous system. To date, there is no strong evidence to support the suggestion that prion disease can be acquired by receiving urinary gonadotropins. However, inoculation experiments using hamsters demonstrated that inoculation with urinary PrPSc led to its accumulation in brain tissue, suggesting that inoculation of urinary PrPSc results in prion infection.

The acceptability of urine as a source of medicine is affected by the occurrence of prion diseases in the country from which the urine is collected. In the United Kingdom, uFSH-HP has been withdrawn because of Creutzfeldt-Jakob disease in Italy, where the urine for this gonadotropin preparation originates. Based on studies of prion clearance from plasma fractions, it has been estimated that almost all of the prion proteins in urine can be coprecipitated and removed with inorganic salts during the manufacturing process. In this study, we demonstrated that prion proteins can be detected in hMG preparations by MS, suggesting that there is potential risk for developing prion disease in using hMG preparations. As described above, many clinicians still prefer to use hMG preparations to add LH activity during follicular stimulation. Although there is no reason to doubt the safety of commercially available urinary gonadotropin preparations at present, the availability of recombinant alternative gonadotropins including FSH and LH (or hCG) will make it likely that the use of urine as a source for gonadotropins will become obsolete in the near future.

In conclusion, we identified nongonadotropin urinary protein contaminants in hMG preparations that may affect the properties of these preparations. Both uFSH-HP and rFSH are highly purified, and differences in the quantities of the α subunit isoforms are considered responsible for the observed differences in FSH activity.

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References


From the Department of Obstetrics and Gynecology and Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan.

Address correspondence to: Yoshimitsu Kuwabara, M.D., Ph.D., Department of Obstetrics and Gynecology, Nippon Medical, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan (kuwa@nms.ac.jp).

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