Assessment of the Immunogenicity of gonadotrophins during Controlled Ovarian Stimulation

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Controlled Ovarian stimulation (COS)

- Controlled ovarian stimulation (COS) is an essential part of the in vitro fertilization (IVF) techniques.
- IVF procedures have historically used protocols involving administration of gonadotrophins to increase the number of oocytes available.
- As with all therapeutic proteins treated subjects may develop an unwanted immune response.
Gonadotrophins

- Gonadotrophins are a family of glycoprotein hormones.
  - Produced at the anterior pituitary gland.
  - Include:
    - **FSH** - follicle-stimulating hormone.
    - **LH** – luteinizing hormone.
    - **hCG** – human chorionic gonadotrophin.
  - Gonadotrophins are heterodimer with two chains, α and β.
  - α chain is common to all Gonadotrophins.
  - β chain confers specificity to each hormone.
  - TSH (Thyroid-stimulating hormone), a pituitary hormone, also shares the same α chain.
Assay strategy

• The presence of a common chain in the gonadotrophins represents a challenge in the evaluation of their immunogenicity. The generation of antibodies against α chain of one hormone should result the presence of antibodies against the rest of gonadotrophins.

• Also, immune response to one hormone may trigger antibodies against the specific β chain of other hormones by epitope spreading.
Assay strategy

A multi-tiered approach was used for the detection of binding antibodies:

1. **Screening assay** (rapid and sensitive) applied to all samples.
2. Positive samples on the Screening assay were analysed in a **Confirmatory assay** (more specific, Immunodepletion).
3. Positive samples after the confirmatory assay were **Titrated**.
4. Positive samples for one hormone were tested for **cross-reactivity** against the other hormones in a competitive assay.
5. Cross-reactivity against **TSH** was also evaluated.
Assay strategy

- The developed methods were a Bridging format.
- The detection was by Electrochemiluminescence (ECLA) on a Mesoscale Apparatus.
- The relative light units (RLU) were transformed to Binding Index (BI) by dividing the response of each sample by the response obtained from a blank serum.
Generation of Tracers

• FSH, LH and hCG were labelled with biotin and SulfoTAG.
• SulfoTAG labelled hormones were prepared according to the protocol provided by Mesoscale.
• Biotinylation of hormones was performed with Sulfo-NHS-LC-Biotin following Thermo Fisher Scientific instructions.
Generation of Positive controls

- Positive controls were prepared by diluting specific purified commercial antibodies on a pool of sera from healthy women.
- The commercial antibodies were affinity purified polyclonal antibodies.
- Each positive control (for FSH, hCG and LH) were produced by mixing antibodies specific for each $\beta$ chain with and antibody against the common $\alpha$ chain.
- Several antibodies against each $\beta$ chain were tested individually to select the antibodies with appropriate sensitivity and specificity.
Generation of Positive controls. FSH

- Several commercial antibodies from different commercial sources were tested.
- Antibodies against the $\alpha$ and $\beta$ chain of each hormone were tested.
• The specificity of the antibodies was confirmed in a competitive assay.
• Fixed amounts of each antibody, producing a similar response on the assay, were mixed with increasing amounts of FSH.
Generation of Positive controls. LH

- Anti-α chain and anti-β chain antibodies for each hormone were combine to produce a positive control able to recognize the whole molecule.
The specificity / cross-reactivity of the antibodies against each hormone were tested by a competitive assay were variable amounts of un-labelled hormones competed with a fixed amount of antibody and tracer.

**Cross-reactivity / specificity of anti-LH α antibody**

**Cross-reactivity / specificity of the anti-LH β antibody**

**Cross-reactivity / specificity of anti-LH positive control (α+β)**
Confirmation of assay specificity, Protein G sample treatment

In order to confirm the specificity of the assay, positive controls and real samples were analysed after treatment with protein-G beads to remove antibodies from the sample.

### Patient Samples results (BI)

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Screening (undisturbed)</th>
<th>Diluted 1:2 with buffer D</th>
<th>Treated with Protein G</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.9</td>
<td>1.5</td>
<td>1.6</td>
</tr>
<tr>
<td>8</td>
<td>3.7</td>
<td>2.7</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Cross-reactivity between hormones

• Due to the presence of the common α chain and the homology on the β chain, the determination of the cross-reactivity was determined for all positive samples.

\[ \% \text{Cross} = \left( \frac{\% \text{ of Difference with the related hormone}}{\% \text{ of Difference on confirmatory assay (with FSH)}} \right) \times 100 \]

<table>
<thead>
<tr>
<th>Sample</th>
<th>NO Competitor</th>
<th>FSH, Conf. assay</th>
<th>TSH</th>
<th>LH</th>
<th>hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Screening assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LRU</td>
<td>BI</td>
<td>Result¹</td>
<td>RLU</td>
<td>%Diff</td>
</tr>
<tr>
<td>a</td>
<td>53.5</td>
<td>1.8</td>
<td>Positive</td>
<td>26.5</td>
<td>50.5 %</td>
</tr>
<tr>
<td>b</td>
<td>89.5</td>
<td>3.2</td>
<td>Positive</td>
<td>27.0</td>
<td>69.8 %</td>
</tr>
<tr>
<td>c</td>
<td>43.0</td>
<td>1.3</td>
<td>Positive</td>
<td>33.0</td>
<td>23.3 %</td>
</tr>
</tbody>
</table>

(1) Screening cutpoint 1.3BI
(2) Using a 41.3% of difference as a confirmatory cutpoint
Methods validation. Sensitivity and cutpoint

- Screening cutpoint was calculated as the response (as BI) resulting in a 5% false positive rate when serum samples from 50 healthy un-treated women were analysed in the assay.
- Confirmatory assay cutpoint was calculated with a 1% false positive rate.
- The sensitivity was established at the concentration resulting in a response at the screening cutpoint of each assay, with a 95% consistency.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (ng/mL)</th>
<th>Screening cutpoint (BI)</th>
<th>Confirmatory cutpoint (%Diff)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH</td>
<td>8.18</td>
<td>1.3</td>
<td>41.3</td>
</tr>
<tr>
<td>LH</td>
<td>14.33</td>
<td>1.3</td>
<td>31.1</td>
</tr>
<tr>
<td>hCG</td>
<td>15.42</td>
<td>1.1</td>
<td>19.7</td>
</tr>
</tbody>
</table>
Methods validation. Precision

• The precision of the screening and confirmatory and titration assays was studied.

• The precision of the method was studied by analysing positive controls, prepared at three different concentrations, QCNeg, QCLow, QCMedium and QCHigh.

• The QCs were analysed in sextuplicated in 6 independent assays, by two different technicians analysed in a minimum of three days.

• Intra-assay and Inter-assay precision was calculated (%CV).

• In all the case the %CV was below 15%.
Methods validation. Stability

• QCLow and QCHigh were analysed in triplicated after several storage conditions.

• Results were compared with the results obtained from QC samples stored undisturbed at -80°C.

• The following conditions were studied:
  ✓ 4 hours at room temperature
  ✓ 24 hour at +4°C
  ✓ After 3 freeze/thaw cycles at -20°C
  ✓ Long term at -20°C up to 12 months was studied for anti-LH and anti-hCG antibodies
  ✓ For FSH stability at -20°C was studied up to 3.5 years
Methods validation. Matrix Effects

• QCLow and QC Medium samples were prepared in buffer, pooled human serum, 10 individual normal sera and 5 human haemolysed sera.

• Results obtained for the 10 individual normal sera and the 5 haemolysed sera were ±20% the response obtained in pooled sera.
Methods validation. Drug Tolerance

• The drug tolerance of the assay was studied adding increasing concentrations of hormones on the screening assay. The QCLow validation and the QCMedium were spiked with different concentration of unlabelled hormone.

• The lowest concentration of hormone that produced a response just below the cut point was considered the drug tolerance.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Drug Tolerance (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH</td>
<td>470</td>
</tr>
<tr>
<td>LH</td>
<td>230</td>
</tr>
<tr>
<td>hCG</td>
<td>1880</td>
</tr>
</tbody>
</table>
Methods validation.
System suitability controls

- The following System suitability controls were established for the analysis of samples:
  - QCNeg - below cutpoint
  - QCLow - at or above cutpoint
  - QCMed and QCHigh – in the interval determined during validation from the precision experiments, with a 1% rejection rate.
Clinical studies

- The method to assess immunogenicity of Gonadotrophins was applied to two different clinical studies.


2. A safety and efficacy study comparing a new hMG formulation to a reference product in patients undergoing ovarian stimulation for in vitro fertilization (Eudrat No: 2010-021021-13). hMG includes FSH activity plus LH/hCG activity at a 1:1 ratio.
FSH study

• A total of 27 healthy volunteers were treated with FSH, of whom 24 completed two cycles of treatment.

• The FSH mean daily dose was 150-225 IU for 9-11 days, with a wash out period of 2 months between the two cycles.

• Seven subjects reported at least one adverse event, but none of them was related to treatment.

• Tolerability at the injection site was very good with only one subject reporting mild itching on Cycle 1 and another subject reporting moderate pain and redness on cycle 2.
FSH study. Immunogenicity results

- From the 27 subjects, 148 serum samples were obtained.
- From the 148 samples analysed, 14 were positives after the screening assay.
- Eight samples were positive after the confirmatory assay.
- These eight samples came from two subjects. Both subjects had positive results at the beginning of the study.
- Therefore, no subjects seroconverted during the study.
- Nonetheless, the responses obtained from the positive samples on the screening assay were very low, below 4 BI.
hMG study

• Patients were treated with a mean hMG(FSH+LH) dose of 150-225 IU for 10 days per cycle.
• Patients from two different sites, who did not get pregnant during the first cycle were offered a second cycle after a wash out period of at least 1 month.
• The 25 patients who accepted were analysed for the presence of antibodies anti-FSH, anti-LH and anti-hCG.
• hMG resulted to be very well tolerated with no persistent redness, swelling or itching reported.
hMG study. Immunogenicity results.

- **Anti-FSH antibodies**
  - In total, 126 samples were analysed from 25 patients.
  - From the 126 samples, 3 were positives after screening assay.
  - No positive samples resulted from the confirmatory assay.

- **Anti-LH antibodies**
  - From the 126 samples, 10 samples were positive after the screening assay.
  - No positive samples resulted from the confirmatory assay.
hMG study. Immunogenicity results.

• Anti-hCG antibodies
  ✓ From the 126 samples analysed from 25 patients, 20 samples were positives after the screening assay.
  ✓ After confirmatory assay 7 samples from 3 different patients were found positives.
  ✓ One patient was positive at the beginning of the study.
  ✓ For the two additional patients, the last sample obtained at the end of the study was negative.
  ✓ Therefore, no patients were positive at the end of the study.
Conclusions

• The methods to determine the presence of anti-FSH, anti-LH and anti-hCG antibodies in FSH or hMG treated patients were developed and validated.

• The detection of antibodies was based on a bridging ECL assay on a Mesoscale Discovery platform.

• Due to the presence of a common chain in the different hormones, an strategy to detect cross-reactive antibodies, based in a competitive assay, was used.

• The results obtained from two different clinical studies indicated that the immunogenicity of FSH and hMG, used for Controlled Ovarian Stimulation, was very low. No cross-reactivity beyond the expected level was detected.
Clinical studies

The results presented were published in the American Journal of Reproductive Immunology

Assessment of the immunogenicity of gonadotrophins during controlled ovarian stimulation

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[Link to the article]
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