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SHORT COMMUNICATION

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Assessment of the immunogenicity of gonadotrophins during controlled ovarian stimulation

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Problem: Gonadotrophin hormones are used for the controlled ovarian stimulation (COS) as part of the in vitro fertilization techniques. Therapeutic proteins have the potential to induce an unwanted immune response.

Method of study: The presence of anti-FSH, anti-LH and anti-hCG antibodies were determined in patients from two different clinical trials after the repeated administration of hMG or FSH.

Results: In the first study, 27 subjects were screening for the presence of anti-FSH antibodies. From the 27 patients, only one patient showed the presence of low levels of antibodies. In a second study, 25 patients were screened for the presence of anti-FSH, anti-LH and anti-hCG antibodies. At the end of the study, no patients showed the presence of antibodies.

Conclusion: The results of this study suggest that repeated treatment cycles with FSH or hMG in patients undergoing COS for in vitro fertilization can be safely and effectively applied without concerns for immunogenicity.

KEYWORDS
ADA, antibodies, FSH, hCG, LH

1 | INTRODUCTION

Controlled ovarian stimulation (COS) is an essential part of the in vitro fertilization (IVF) techniques used in the treatment of infertility, because pregnancy and live birth rates are correlated with the number of fertilized oocytes.[1] IVF procedures have historically used protocols involving administration of gonadotrophins to increase the number of oocytes available for eventual embryo transfer.

Gonadotrophins are a family of glycoprotein hormones produced at the anterior pituitary gland which includes follicle-stimulating hormone (FSH), luteinizing hormone (LH) and human chorionic gonadotrophin (hCG).

Subjects treated with therapeutic proteins may develop an unwanted immune response to these products. The consequences of an immune reaction to a therapeutic protein may range from transient appearance of antibodies without any clinical consequences to severe life-threatening conditions.

There is limited information on the occurrence of anti-gonadotrophin antibodies in women undergoing treatment for infertility, but compared to other therapeutic proteins, FSH is considered to have a low immunogenicity potential.[2,3]To monitor the presence of antibodies against FSH, LH and hCG on gonadotrophin-treated patients, a testing strategy was designed following the current guidelines and published recommendations.[4-7] The detection of antibodies in this study was based on the electrochemiluminescence (ECL) assay. Due to the presence of a common chain in the gonadotrophin hormones, the testing strategy also included a determination of the cross-reactivity of the antibodies against each gonadotrophin.

In summary, the aim of this study was to determine the putative presence of antibodies to FSH, LH and hCG in patients treated with

FSH or hMG undergoing controlled ovarian stimulation (COS) for in vitro fertilization.

2 | MATERIALS AND METHODS

2.1 | Clinical trials

2.1.1 | Patients

The first study (code 11E/FSH03, Eudract No: 2012-000269-19) [8] was a prospective, open-label, single-arm, immunogenicity study in healthy subjects undergoing (controlled ovarian hyperstimulation) COH for oocyte donation treated with FSH (Fostimon, IBSA Institut Biochimique SA). The primary end point of the study was to determine the presence of anti-FSH antibodies. The safety end point was to determine adverse events and tolerability reactions that could be linked to an immunological reaction, such as immediate or delayed hypersensitivity at the injection site or manifestations of systemic hypersensitivity. Serum samples were drawn from each volunteer at screening visit, at Cycle 1 (visits 2, 3 and 4) and at Cycle 2 (visits 5, 6 and 7). The clinical study was performed at the *Institut Universitari Dexeus*, *Barcelona*, *Spain*.

The second study (code 10EU/HMG02, Eudract No 2010-021021-13) [9] was a safety and efficacy study comparing a new hMG formulation (Meriofert®, IBSA Institut Biochimique SA, Pambio-Noranco, Switzerland) to a reference product (Menopur®, Ferring Pharmaceuticals, Saint-Prex, Switzerland) in patients undergoing ovarian stimulation for in vitro fertilization (IVF). Patients treated in two centres who did not get pregnant during the study were offered the opportunity to perform a second cycle of treatment with Meriofert. Serum samples were drawn from each patient at screening visit (Visit 1, baseline), on the day of oocyte pick-up (Visit 4, OPU) and at the end of treatment (Visit 6, b-hCG test). Patients who did not get pregnant may start a second treatment cycle, and serum samples were obtained at the same time points.

In study 10EU/HMG02, the presence of antibodies against FSH, LH and hCG was determined.

Both studies were conducted in accordance with principles of good clinical practice and were approved by the appropriate institutional review boards and regulatory agencies. Written informed consent was provided by all subjects.

2.2 | Assessment of immunogenicity

2.2.1 | Preparation of tracers

Follicle-stimulating hormone and human chorionic gonadotrophin were provided by IBSA Institut Biochimique S.A., and LH was purchased from Fitzgerald Industries International (Acton, MA, USA) and TSH from Calbiochem (Merk Millipore, Darmstadt, Germany). Biotinylated and Sulfo-TAG-labelled hormones were prepared according to the instructions provided by MesoScale Dyscovery [10] and Thermo Fisher Scientific.[11]

2.2.2 | Antibody detection protocol

Positive controls for the assays were prepared by diluting commercial antibodies on the pool of sera from healthy woman. The antibodies were anti-human hCGα antibody, reactive against alpha chain of hCG, FSH, LH and TSH (AbCam, Cambridge, UK), anti-human FSHβ antibody and anti-human hCGβ antibody (LSBio, Seattle, WA, USA) and anti-human LHβ antibody (Fitzgerald, Acton, MA, USA).

For the screening assay, the samples and controls were mixed in a polypropylene 96-well plate with a solution containing biotinylated hormone and Sulfo-TAG-labelled hormone and incubated for 1 hour. Twenty-five μL from each well in the polypropylene plate was transferred to the streptavidin plate and incubated for 1.5 hour. Streptavidin plate was washed. Read buffer T (MSD, Rockville, MD, USA) was added to the plate, and the plate was read on the Sector Image 2400 Instrument (MSD, Rockville, MD, USA). The relative light units (RLU) for each sample or control were recorded, and the Binding Index (BI) was calculated as the ration between the RLU for each sample/control and the RLU of the blank control.

The confirmatory assay was a competitive assay, performed as described for the screening assay, but each sample was analysed twice, with and without competitor (non-labelled hormone). After analysis, the percentage of difference between the samples analysed with and without competitor was calculated. If the response seen on the screening assay was specific, the presence of a large amount of unlabelled hormone should abolish the response on the assay.

The strategy for the detection of antibodies in this study followed a multitier approach: only samples found positives on the screening assay were submitted to the confirmatory assay.

Samples found positives after the confirmatory assay were submitted to a titration assay. The titre of the sample was defined as the last dilution showing a result above cut point.

The cross-reactivity of the positive samples against related hormones was assessed by performing a confirmatory assay using the related hormone as a competitor. If antibodies present in the sample cross-reacted against the related hormone, the presence of unlabelled related hormone should reduce the response in the assay.

2.2.3 | Method validation

The methods to detect antibodies against FSH, LH and hCG were validated following the current guidelines by the FDA[4] and EMA [5] and the published recommendations.[6,7,12] The validation included determination of the cut point, sensitivity, specificity, precision, matrix effects and stability.

The screening cut point for each assay was determined at the 95 percentile of the distribution obtained from 50 serum samples from untreated healthy women. The screening cut points were 1.3BI for FSH, 1.3BI for LH and 1.1BI for hCG. For the confirmatory assays, the cut points were calculated as the 99 percentile of the %Diff obtained from 50 serum samples from untreated healthy women. The confirmatory cut point was 41.3%Diff for FSH, 31.1%Diff for LH and 19.7%Diff for hCG.

The sensitivity of the assays was determined by serially diluting the control antibody in pooled serum from healthy donors. The sensitivity for the FSH assay was 8.18 ng/mL for LH 14.33 ng/mL and for hCG 15.42 ng/mL.

The precision of the screening and confirmatory assays for the three anti-hormone assays was determined by analysing three QC samples with different levels of anti-hormone antibodies. The precision, measured as %CV, was below 15%CV for all the QC samples.

The stability of anti-hormone antibodies in human serum was determined at two different antibody levels (high and low). The samples were stable when submitted to 3 freeze-thaw cycles, 4 hours at room temperature and 24 hours at 4°C.

2.2.4 | Study sample analysis

Serum samples were analysed in duplicate. Sample results with duplicates with %CV >15% were discarded and re-analysed. The samples were analysed in batches (ie assay runs or plates), and each screening assay run included a set of negative (QCneg) and positive (QClow, QCmedium and QChigh) system suitability controls. Additionally, each confirmatory assay run included a set of QC (QCconf) analysed with and without competitor.

3 | RESULTS

3.1 | Study 11E/FSH03

3.1.1 | Patient characteristics and disposition

A total of 41 female healthy volunteers were screened, of whom 27 started the treatment and 24 completed the two treatment cycles.

3.1.2 | Adverse events

Seven subjects reported at least an adverse event, but none of them was related to the treatment. All the AEs were classified as mild and not related to study drug. Only one serious adverse event (SAE), considered not related to the study treatment, was reported by one subject.

3.1.3 | Local tolerance

Side effects related to tolerability at administration site were collected at each visit. This included the incidence of local injection site reactions such as pain, persistent redness, swelling and itching. Tolerability at injection site resulted to be very good, with only one subject reporting mild itching after the first injection on Cycle 1—Visit 2, and another subject reporting moderate pain and redness, after the first injection of Cycle 2—Visit 5.

3.1.4 | Immunogenicity

In total, 27 subjects starting the treatment were analysed to detect antibodies against FSH. From those 27 patients, 148 serum samples were obtained and analysed. From the 148 samples analysed, 14 samples were positive after the screening assay.

From those 14 positive samples in the screening assay, eight samples were positive after confirmatory assay (5.4%). These eight samples came from two subjects; both subjects had positive results at the beginning of the study (V2). Therefore, no subjects seroconverted during the study. From the two subjects with positive results, one had only positive results until V3 and one had positive results until the end of the study (V7). Therefore, after treatment, only one volunteer showed positive results. The titre for those positive samples was dilution 2 for two samples, dilution 4 for five samples and dilution 8 for one sample.

The cross-reactivity against TSH, LH and hCG was determined in those eight samples. Regarding the cross-reactivity against TSH, one sample showed no cross-reactivity with TSH. From the seven samples showing cross-reactivity, the percentage was ranged from 4.3% to 41.2%. Regarding the cross-reactivity against LH, two samples showed no cross-reactivity with LH. From the six samples showing cross-reactivity, the percentage was ranged from 7.9% to 22.1%. Regarding the cross-reactivity against hCG, the eight positive samples showed cross-reactivity with hCG with percentages ranged from 3.9% to 50.5%. These levels of cross-reactivity were expected because FSH, TSH, LH and hCG share a protein chain.

It is noteworthy to mention that the responses obtained from the positive samples were low (maximum response in the screening assay = 4 BI) compared to the cut point (1.3 BI). According to the published guidelines, and based on the data from clinical trials, the concentration of antibodies associated with clinical events was 250–500 ng/mL. This antibody concentration should produce in our assay a response between 24 and 50 BI, as shown during assay validation, far from the 4 BI maximum response obtained in this study. Given the very high sensitivity of the assay and the low responses obtained, those positive results are to be considered not clinically relevant.

3.1.5 | Efficacy: ovarian stimulation

The same total quantity of drug was used to obtain an equivalent number of oocytes in the first and the second cycle, 202.8 IU/oocyte for Cycle 1 and 163.0 IU/oocyte for Cycle 2 (*P* value .12). The mean daily dose resulted to be statistically significantly higher in the second cycles, 186.0 IU for Cycle 1 and 201.4 IU for Cycle 2 (*P* value .004). However, as the quantity of drug needed to retrieve one oocyte was equivalent between the first and the second cycle, the difference in the daily dose is considered non-clinically significant.

3.2 | Study 10EU/HMG02

3.2.1 | Patient characteristics

Patients from two sites who did not get pregnant during the study were offered the opportunity to perform a second cycle of treatment with hMG-IBSA. The 25 patients who accepted were analysed for the presence of antibodies anti-FSH, anti-LH and anti-hCG.

3.2.2 | Adverse events and local tolerance

hMG resulted to be very well tolerated with no persistent redness, swelling or itching reported. Additional data, including the efficacy results, have been reported in a separate article[13] (accepted for publication in Reproductive Biomedicine Online).

3.2.3 | Immunogenity

Anti-FSH antibodies

Twenty-five patients were analysed to detect antibodies against FSH. From the 25 patients, 126 samples were obtained and analysed. From those 126 samples, three samples were positive after the screening assay.

From those three positive samples in the screening assay, FSH confirmatory assay did not show any positive sample with confirmatory results above the confirmatory cut point.

In conclusion, at the end of the study, all the samples analysed were negative for the presence of anti-FSH antibodies. Therefore, no patients developed an anti-FSH antibody response during the study.

Anti-LH antibodies

Twenty-five patients were analysed to detect antibodies against LH. From the 25 patients, 126 samples were obtained and analysed. From those 126 samples, 10 samples were positive after the screening assay. From those 10 positive samples in the screening assay, LH confirmatory assay did not show any positive sample with confirmatory results above the confirmatory cut point.

In conclusion, at the end of the study, all the samples analysed were negative for the presence of anti-LH antibodies. Therefore, no patients developed an anti-LH antibody response during the study.

Anti-hCG antibodies

As before, 25 patients were analysed to detect antibodies against hCG.

From the 25 patients, 126 samples were obtained and analysed. From those 126 samples, 20 samples were positive after the screening assay with a maximum response of 1.8 BI.

From those 20 positive samples in the screening assay, hCG confirmatory assay showed positive results for seven samples.

Therefore, in the case of anti-hCG antibodies, 119 samples were negative and seven samples were positive after the confirmatory assay, leading to 5.6% of positive samples for anti-hCG binding antibodies.

The titre for those seven samples was dilution 1 for two samples, dilution 8 for three samples and dilution 16 for two samples.

After the analysis of those seven positive samples for cross-reactivity against TSH, six samples showed some level of cross-reactivity, as expected due to the presence of a common beta chain in hCG and TSH molecules, and one sample showed no cross-reactivity with TSH. From the six samples showing cross-reactivity, five had percentages between 33.6% and 60.3% and one additional sample showed cross-reactivity higher than 100% (ie 231.9%) meaning that, in this case, the TSH was able to inhibit the response in the assay better than hCG. Nevertheless, the results for that sample (screening assay 1.2BI and titre 1) were very low, so the cross-reactivity results should be taken with precaution.

From the 25 patients analysed, 22 had all the samples negative and three patients had, at least, one positive result. From the three patients with positive results for anti-hCG antibodies, one already had a positive result at the beginning of the study. Therefore, only two patients who were negative at the beginning of the study showed a positive result during the study, although the response obtained for those samples was very low or borderline. Additionally, the last sample obtained for those two patients was negative for the presence of anti-hCG antibodies. Consequently, at the end of the study, no patients were positive for the presence of anti-hCG antibodies and no patients seroconverted. The presence of a low positive result in the middle of the study, which was not confirmed in the last sample from the same patient, may be due to several reasons: (i) the positive result was a borderline result which, due to the normal variability of the assay, was not maintained in the final sample, or (ii) the positive result was a false-positive result. It is noteworthy that the assays for the detection of antibodies used in this study are developed to minimize the false-negative rate by introducing a theoretical 1% false-positive rate.

In summary, the results obtained for the seven positive samples indicate that the amount of ADA was very low, with the highest responses, both in the screening assay and the titre, seen on the patient who already had a positive result at the beginning of the study. The two patients with a positive result during the study showed borderline responses, with screening assay results 1.2BI and titres 1, the lowest possible response, which may be due to false-positive results.

It should be noted that the anti-hCG assay has a very low screening cut point and a very high sensitivity (15.42 ng/mL), and therefore, low positive results in this assay will be not clinically relevant.

In any case, at the end of the study, no patients showed the presence of anti-hCG antibodies.

4 | DISCUSSION

In the present study, the presence of antibodies against FSH, LH and hCG was determined in patients after the repeated administration of hMG or FSH.

Therapeutic proteins have the potential risk of antibody formation on the treated patients, reducing the safety and efficacy of the product.[6] Structural, functional and animal data are generally not adequate to predict immunogenicity in humans. Therefore, the immunogenicity of the proposed product has to be assessed in humans. Therefore, we developed a highly sensitive method to detect putative antibodies against FSH, LH and hCG. The methods were validated following the latest guidelines and recommendations.[4–7,12]

Follicle-stimulating hormone is a 35.5-kDa glycoprotein heterodimer, consisting of two polypeptide chains, alpha and beta. The structure of FSH is very similar to those of luteinizing hormone (LH), thyroid-stimulating hormone (TSH) and human chorionic gonadotrophin (hCG). The alpha subunits of LH, FSH, TSH and hCG are almost

identical and contain around 96 amino acids, while the beta subunits are specific to each hormone. FSH has a beta subunit of 111 amino acids (FSH β), which confers its specific biologic action.[14] LH and hCG have beta subunits of 120 and 145 amino acids, respectively, that confer its specific biologic action Due to the presence of a common chain and the homology between FSH, LH and hCG, the putative antibodies directed against one hormone could cross-react with the other hormones.

Some studies have shown the presence of anti-FSH antibodies (IgG, IgM and IgA) on healthy non-pregnant women and a decrease in IgG and IgM on uncomplicated pregnancy [15] and an increase in those antibodies on infertile women.[15,16] The levels of anti-FSH IgM antibodies were associated with peripheral FSH levels on patients with tubal and male factor infertility.[17] According to these authors, the anti-FSH IgA antibodies detected in serum could be part of the mucosal response involved in the induction of immune tolerance to seminal constituents,[18] as FSH is also present in semen. In those studies, the infertile patients were indicated for IVF, but serum samples were obtained before the administration of exogenous FSH.[16] Anti-FSH antibody levels were elevated both on patients who had previously undergone IVF procedures and patients who had never undergone IVF.

As opposed to other recombinant hormones, FSH, LH and hCG in the drug used in the present study are from human origin; therefore, immune responses to those hormones might be prevented by self-tolerance mechanisms as the protein is not recognized as "non-self". Consequently, it is not surprising that the immunogenicity detected in those studies was very low.

The results obtained in the present study demonstrated that the administration of FSH or hMG did not induce a significant increase in the immune response to FSH, LH or hCG, measured by the presence of antibodies. Also, in addition to the lack of antibody responses, in the present study were no treatment-related hypersensitivity reactions and tolerability at the injection site was very good. The efficacy of the FSH during the first and the second cycle of treatment in study 11E/FSH03 was equivalent, because the same total quantity of drug was used to obtain an equivalent number of oocytes, indicating that the drug was not neutralized after repeated treatment.

In summary, the results of the present study suggest that repeated treatment cycles with FSH or hMG in patients undergoing COS for in vitro fertilization can be safely and effectively applied without concerns for immunogenicity.

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