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Two human menopausal gonadotropin preparations display different early signalling *in vitro*

Running title. Comparison between two hMGs

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Abstract

Commercial human menopausal gonadotropin drugs are marketed for the treatment of infertility and consist of highly purified hormones acting on receptors expressed in target gonadal cells. Menopur® and Meriofert® are combined preparation of FSH and hCG and are compared *in vitro* herein. To this purpose, the molecular composition of the two drugs was analyzed by immunoassay. The formation of FSH receptor and LH/hCG receptor (FSHR; LHCGR) heteromer, intracellular Ca^{2+} and cAMP activation, β-arrestin 2 recruitment and the synthesis of progesterone and estradiol were evaluated in transfected HEK293 and human primary granulosa lutein cells (hGLC) treated by drugs administered within the pg-mg/ml concentration range. Molecular characterization revealed that Meriofert® has a higher FSH:hCG ratio than Menopur® which, in turn, displays the presence of LH molecules. While both drugs induced similar FSHR-LHCGR heteromeric formations and intracellular Ca^{2+} increase, Meriofert® had a higher potency than Menopur® in inducing a cAMP increase. Moreover, Meriofert® revealed a higher potency than Menopur® in recruiting β-arrestin 2, likely due to different FSH content modulating the tridimensional structure of FSHR-LHCGR-β-arrestin 2 complexes, as evidenced by a decrease in bioluminescence resonance energy transfer (BRET) signal. This drug-specific activation of intracellular signaling pathways is consistent with the molecular composition of these preparations and impacts downstream progesterone and estradiol production, with Menopur® more potent than Meriofert® in inducing the synthesis of both the steroids. These findings are suggestive of distinct *in vivo* activities of these preparation, but require cautious interpretation and further validation from clinical studies.

Introduction

Preparations of human menopausal gonadotropins (hMGs) were developed for infertility treatment and contain both FSH and LH activites. However, the "LH activity" of these drugs is mostly due to prevalent hCG (Lehert *et al.*, 2010; Ezcurra and Humaidan, 2014), rather than LH molecules (van de Weijer *et al.*, 2003). Depending on purification methods and production procedures, commercial hMG preparations may contain hCG of pituitary (Menopur®; Ferring SA, Saint-Prex, Switzerland) or trophoblast origin (Meriofert®; IBSA Institut Biochimique SA, Lugano, Switzerland), providing the potential for different glycosylation patterns and distinct biological activities (Fournier, 2016).

In the last decade, it was demonstrated that LH and hCG mediate different intracellular signaling pathways in various cell models *in vitro* (Casarini *et al.*, 2012, 2016c, 2018a; Gupta *et al.*, 2012; Riccetti *et al.*, 2017d) due to specific contact points with their G protein-coupled receptor, the luteinizing hormone/chorionic gonadotropin receptor (LHCGR) (Grzesik *et al.*, 2014, 2015). Moreover, commercial hMGs have unique glycosylation profiles which are linked to preparation-specific patterns of intracellular signaling cascades (Riccetti *et al.*, 2017a). Therefore, distinct gonadotropin-dependent signatures associated with hCG:LH ratio and source of hCG/LH activity may be supposed. The combined presence of FSH with hCG and LH might potentially result in cross-talk of intracellular signaling cascades and modulate the cell responses (Casarini *et al.*, 2016d). The FSH receptor (FSHR) and LHCGR-mediated pathways overlap in large part and, once activated by their cognate ligands, consist in cAMP/protein kinase A (PKA) activation linked to steroidogenic, as well as proliferative and apoptotic signals (Zhang *et al.*, 2009; Casarini *et al.*, 2016d). cAMP/PKA-pathway activation is temporarily followed by β-arrestin 2 recruitment at the FSHR/LHCGR, resulting in receptor internalization (Reiter *et al.*, 2017). FSHR and LHCGR may physically interact forming heteromers in the cell membrane (Ji *et al.*, 2004; Feng *et al.*, 2013; Mazurkiewicz *et al.*, 2015; Jonas *et al.*, 2018), which modulate gonadotropin-dependent

intracellular signaling pathways (Zhang *et al.*, 2009), in cell co-expressing both the receptors such as granulosa cells (Casarini *et al.*, 2018b).

hMGs are administered during the ovarian stimulation phase of ART to achieve multi-follicular maturation assuming that the addition of LH/hCG to the FSH activity may improve the clinical outcome, measured as number of oocytes retrieved, embryo quality and live birth rate (Casarini *et al.*, 2016a). To date, despite recent ESHRE guidelines (Ovarian Stimulation *et al.*, 2020), there is still no clear *consensus* on the best ovarian stimulation protocol to be applied to women undergoing ART, with the choice of gonadotropins regimen, including recombinant and urinary formulations, based on the clinicians' decision. Most of the clinical studies aimed to compare the efficacy of recombinant *versus* urinary preparations in influencing ART outcome *in vivo*, and contrasting data are reported in the literature (Cantineau *et al.*, 2007; Ezcurra and Humaidan, 2014; Santi *et al.*, 2017a). Thus, dissecting the *in vitro* activities of gonadotropin hormone preparations in eliciting LHCGR- and/or FSHR-signal activation may aid deciphering *in vivo* outcomes.

This study therefore aimed to compare the *in vitro* activities of two hMG preparations that are commonly used in ART procedures- Menopur® and Meriofert®. Using a combination of gonadotropin hormone receptor transfected HEK293 cell lines and primary human granulosa-lutein cells, we tested the effects of these two hormone preparations on LHCGR-FSHR heteromers, cAMP production, β-arrestin 2 recruitment and steroidogenesis.

Materials and Methods

Gonadotropin commercial preparations

Two hMG preparations, Menopur® 75 international units (IU; Ferring Pharmaceuticals, Saint Prex, Switzerland) and Meriofert® 75 IU (IBSA Institut Biochimique SA), as well as recombinant hCG (r-hCG) Ovitrelle® 250 µg/0.5 ml (Merck KGaA, Darmstadt, Germany) and FSH (rFSH) Gonal-F® 1050 IU (Merck KGaA) were analyzed. Recombinant human LH (hLH) Luveris® 75 IU (Merck KGaA) was also used where appropriate.

Cell culture and transfection

HEK293 cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine (Thermo Fisher Scientific, Waltham, MA, USA). Transient transfections were performed in 96-well plates, using Metafectene PRO (Biontex Laboratories GmbH, München, Germany), following the manufacturer's instructions and validated protocols (Riccetti *et al.*, 2017a, 2017d; Lazzaretti *et al.*, 2019). Briefly, $3x10^4$ cells were seeded in 96 well-plates, and treated by 200 ng/well plasmids and 0.5 µl/well of Metafectene PRO. Cells were cultured two days before gonadotropin administration.

Human primary granulosa lutein cells (hGLC) were isolated from ovarian follicles of women undergoing oocyte retrieval for ART, as previously described (Casarini *et al.*, 2012, 2016c, 2017; Riccetti *et al.*, 2017a, 2019). Briefly, cells were purified using a 50% Percoll density gradient (GE Healthcare, Little Chalfont, UK) and cultured 5-6 days before experiments, allowing *FSHR* and *LHCGR* expression recovery (Nordhoff *et al.*, 2011). hGLC were cultured at 37°C and 5% CO_2 in DMEM-F12 medium, supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml fungizone (all from Thermo Fisher Scientific). Study approval from the local Ethics Committee was obtained (Nr. 796 19th June 2014, Reggio Emilia, Italy). Donors were anonymous and signed a written consent.

Quantification of FSH and hCG content by immunoassay

These experiments were performed after recalibrating hMGs, which are supplied as international units, IU, using recombinant FSH and hCG as *in vitro* standards. This procedure was previously described as an extrapolation of FSH and hCG (and LH) against recombinant gonadotropins after immunometric measurement. Although this method allows to determine the composition of menotropins, it has some limitations, above all the assumption that the antibody used in the assay has same binding affinity to all the FSH, hCG and LH glycosylation variants. Indeed, IU are a measure of the *in vivo* activity of these molecules, obtained by mouse/rat bioassay (Steelman and Pohley, 1953; Bangham and Grab, 1964), and are not indicative of the number of hormone molecules. Therefore, they are not suitable for *in vitro* experiments. *In vitro* comparisons between Menopur and Meriofert were carried out as mass/volume (μg/ml), since IU are indicative of the hormone activity *in vivo*, in rodents (Steelman and Pohley, 1953; Bangham and Grab, 1964). Therefore, preparations were re-calibrated against recombinant FSH and hCG, which were quantified in both IU and μg/ml and used as internal standards.

FSH, hCG and LH immunoreactivity of commercial preparations was evaluated by immunoenzymatic sandwich assay (Architect System, Abbott, Abbott Park, Illinois, USA; Access Immunoassay Systems; Beckman Coulter Inc., Brea, CA, USA, respectively), as previously described and validated (Riccetti *et al.*, 2017b). Samples calibration was made against the FSH (World Health Organization, WHO, 1st International Standard 92/150) or hCG standard (5th IS; National Institute for Biological Standards and Control, NIBSC, 07/364), following manifacturer's instructions. Briefly, gonadotropins were immobilization on paramagnetic particles coated with either a mouse monoclonal anti-hCGβ or anti-FSHβ antibody in complex with a goat anti-mouse IgG. The rabbit anti-hCGβ alkaline phosphatase conjugate or a mouse anti-FSHα acridinium conjugate was added, which reacts with the immobilized FSH/hCG. From a standard curve, and

chemolumiscent reaction, μg/ml of each hMG preparation was determined (Vankrieken and De Hertogh, 1995).

Evaluation of receptor homo/heteromers, intracellular cAMP production, Ca^{2+} increase and β-arrestin recruitment by bioluminescence resonance energy transfer (BRET)

BRET methods were carried out as previously decribed (Riccetti *et al.*, 2017d, 2017a; Brigante *et al.*, 2019; Lazzaretti *et al.*, 2019; Sperduti *et al.*, 2019). Briefly, intracellular cAMP production was evaluated in HEK293 cells transiently transfected with *LHCGR* and/or *FSHR* encoding plasmids, (with protein expression control evaluated by Western botting, Fig S1), and the BRET-based cAMP sensor cAMP sensor using YFP-Epac-RLuc (CAMYEL) (Jiang *et al.*, 2007; Ayoub *et al.*, 2015). To assess β-arrestin 2 recruitment, HEK293 cells were transiently transfected by plasmids encoding C-terminally rluc-tagged LHCGR (100 ng/well) or FSHR (50 ng/well) and N-terminal yellow fluorescent protein for resonance energy transfer (yPET)-tagged β-arrestin 2. For both the cAMP and β-arrestin 2 assays, HEK293 cells were incubated 30 minutes in 40 μl/well PBS and 1 mM Hepes, in the presence or absence of increasing gonadotropin hormone concentrations (10^{-7}-10^{3} μg/ml). Plasmids encoding the *Renilla reniformis* luciferase variant 8 (Rluc8)- and Venus-tagged receptors were used for evaluating FSHR and LHCGR homo/heteromerisation, by transfecting cells with a fixed amount of Rluc8-tagged receptor encoding plasmid (50 ng/well FSHR/rluc) and increasing LHCGR/venus-tagged receptor encoding plasmid concentrations (0-400 ng/well). Correspondence between amount of plasmid administered to each well and tagged protein produced by cells were determined by Förster resonance energy transfer (FRET), using excitation and emission settings at 470 ± 20 and 530 ± 20 nm wavelength, respectively, and served as controls. These receptors carry also a DYKDDDDK-tag (FLAG) protein tag used for detecting protein expression by Western blotting. Ca^{2+} increase was evaluated in transfected HEK293 cells expressing the BRET aequorin biosensor and pre-incubated for 45 min with Hank's Balanced Salt

Solution (HBSS) supplemented with calcium and magnesium, 1 mM Hepes (both from Thermo Fisher Scientific) and 5 μM of the coelenterazine h substrate (NanoLight Technologies, a division of Prolume Ltd., Pinetop, AZ, USA). The lowest effective fixed concentration of gonadotropin hormone was administered to cells and Ca^{2+} release measured over 200 s. Cells treated with 5 μM of the sarco/endoplasmic reticulum Ca^{2+} ATPase enzyme inhibitor, thapsigargin (Tocris Bioscience), served as positive control (Brigante *et al.*, 2019), while vehicle-treated cells served as negative controls. All BRET measurements were performed using a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany) by detecting light emissions at 480 and 540 nm wavelengths.

Western blotting

Western blotting methods were conducted as previously described (Casarini *et al.*, 2012, 2016c, 2017; Riccetti *et al.*, 2017a, 2019; Brigante *et al.*, 2019; Lazzaretti *et al.*, 2019; Sperduti *et al.*, 2019). Protein lysates obtained from $3x10^5$ cells were resolved using 12% acrylamide gel electrophoresis and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted onto polyvinylidene fluoride (PVDF). Transfection efficiency was determined using antibodies to LHCGR (#PA5-21271; Thermo Fisher Scientific), FSHR (#PA5-28764; Thermo Fisher Scientific) and FLAG (#7425; MilliporeSigma; Burlington, MA, USA). Additional Westerns were conducted for β-arrestin 1/2 antibody (#sc-74591; Santa Cruz Biotechnology Inc.; Dallas, TX, USA) and β-actin normalization control (#A3854; MilliporeSigma). Chemiluminescence was used to determine protein signal (Bio-Rad Laboratories Inc., Hercules, CA, USA) and acquired using the VersaDoc imaging system and QuantityOne software (BioRad Laboratories Inc.).

Evaluation of steroid synthesis

hGLC were seeded in 24-well plates ($5x10^4$ cells/well) and serum-starved 12 h before experiments. Cells were treated for 8 or 24 h with increasing concentrations of gonadotropins (10^{-7} - 10^3 μg/ml). 1 μM 4-androstene-3,17-dione (androstenedione; #A9630; Sigma-Aldrich) was added, where

appropriate, as a substrate to be converted to estrogen by the aromatase enzyme. Reactions were blocked by freezing samples, and total progesterone and estradiol were measured in the cell media by an immunoassay analyser (ARCHITECT 2nd Generation system; Abbot Diagnostics, Chicago, IL, USA), as previously validated (Casarini *et al.*, 2012; Riccetti *et al.*, 2017a, 2019).

Statistical Analysis

BRET data were represented as "induced BRET changes" by subtracting the untreated cells 540/480 nm ratio to values from stimulated cells. Steroid concentrations were represented as ng/ml. Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA) and all results were expressed as means ± SEM for n=4-11 independent experiments. All results were tested for normality using the D'Agostino and Pearson test, then Mann-Whitney's *U*-test, t-test, Kruskall-Wallis or ANOVA followed by post-hoc analysis were performed, as appropriate ($p<0.05$).

Results

Determination of hMG content by immunoreactivity

The potency/concentration of commercial gonadotropin preparations are usually determined in IU by *in vivo* bioassay (Steelman and Pohley, 1953; Bangham and Grab, 1964). As IUs is not representative of the *in vitro* bioactivity, gonadotropin content was determinated in μg/ml. As hMGs are different mixtures of FSH, LH and hCG, gonadotropin hormone content was determined by re-calibrating the drugs against a standard recombinant gonadotropin (Gonal-F® and Ovitrelle®), as previously described (Riccetti *et al.*, 2017a, 2019). Briefly, FSH and hCG immunoreactivity was determined using a monoclonal antibody against the hormone β-subunit, assuming it has similar binding affinity to all gonadotropic hormones composing commercial preparations. To this purpose, FSH, hCG and LH content of samples from Gonal-F®, Ovitrelle® and Luveris® batches were measured by immunoassay and the values obtained (per 5000 mIU-batch diluted in 1 ml) were indicated in a table, together with the activity and mass (mIU/ and ng/ml) declared by the suppliers (Table 1). Menopur® and Meriofert® preparations were analyzed as well and the measured values are expressed in mIU/ml of FSH, hCG and LH international standards (WHO 1st International Standard 92/150 and 5th IS; NIBSC 07/364, respectively). Recombinant FSH, hCG and LH quantification in weight were used to re-calibrate the hMG preparations analyzed, allowing to indicate the FSH, hCG and LH content of Menopur® and Meriofert® preparations as a mass (ng/ml per 5000 mIU-batch).

A 5000 mIU-batch/ml of Menopur® corresponded to 287.4 ± 0.9 ng/ml FSH, 26.1 ± 0.7 ng/ml hCG and 1.2 ± 0.02 ng/ml LH, revealing similar FSH but 2.5-fold higher hCG content than that contained in the same mIU of Meriofert® (285.6 ± 0.5 ng/ml FSH; 10.5 ± 0.5 ng/ml hCG). Interestingly, LH was absent in Meriofert® in contrast to Menopur®. Taken together, these results are indicative of both quantitative and qualitative differences in the gonadotropin hormone content

and composition of Menopur® and Meriofert®.

Evaluation of gonadotropin receptor heteromerisation

Heteromerization of FSHR and LHCGR has recently been reported (Ji *et al.*, 2004; Zhang *et al.*, 2009; Feng *et al.*, 2013; Jonas *et al.*, 2015), with potential roles in signal specificity within the ovulatory follicle. Therefore, to determine the impact of the differences in gonadotropin hormone content of Menopur and Meriofert on FSHR and LHCGR heteromerization, BRET analysis was conducted. Formation of FSHR and LHCGR homomers was also evaluated to check the potential of FSH and hCG of recruiting their specific receptors (Figure 1).

In line with previous reports, constitutive FSHR-LHCGR interactions were observed (Figure 1A), while control experiments in cells co-expressing Rluc8-tagged FSHR and Venus protein alone showed a non-specific interaction as determined by the linear and non-saturating nature of the association (Figure 1B). To evaluate the effect of hMG preparations, FSHR and LHCGR co-expressing cells were treated 10 min with preparations, which were administered as 0.7 µg/ml of the FSH content and corresponding to the effective concentration ~20 nM FSH (Casarini *et al.*, 2014; Riccetti *et al.*, 2019). Menopur® and Meriofert® had no effects on constitutive FSHR/LHCGR heteromerization ($p \geq 0.05$). However, cell treatments with individual gonadotropin hormones at equimolar levels to those in the hMG preparations showed a significant increase in FSHR/LHCGR heteromerization in the presence of either FSH and hCG (Figure 1A) ($p < 0.05$). Control experiments using cells co-expressing Rluc8- and venus-tagged FSHRs or LHCGRs (Figure 1C, D), resulted in increased homomer formation upon treatment by 20 nM of recombinant FSH and hCG compared to the constitutive level ($p < 0.05$) suggesting distinct effects of preparations on both FSHR/LHCGR heteromerization and LHCGR and FSHR homomerization.

FSHR- and LHCGR-mediated intracellular cAMP production and Ca^{2+} increase

Next, we assessed the impact of hMG preparations on signal cascade activation. Preparation-

specific intracellular cAMP production was assessed by BRET in HEK293 cells transiently co-expressing the BRET-based cAMP biosensor CAMYEL together with FSHR (Figure 2A), LHCGR (Figure 2B) or both receptors (Figure 2C, D). All preparations induced dose-dependent cAMP production within the 10^0-10^3 ng/ml concentration range (Table 2). In LHCGR-expressing cells, Meriofert® was ~4-fold more potent than Menopur® in activating cAMP (Figure 2B; $p<0.05$), revealing that the composition of these preparations differently impacts on the intracellular increase of the second messenger. In HEK293 cells transiently co-expressing both the gonadotropin receptors, treatment with Menopur® or Meriofert® induced similar cAMP production ($p\geq0.05$). Interestingly, these two preparations induced right-shifted dose-response curves, compared to recombinant FSH, when they were represented in terms of FSH content (Figure 2C; $p<0.05$), suggesting that the action of hCG/LH at the LHCGR is linked to decreased FSH potency in activating cAMP, in line with previous reports suggesting functional asymmetry within the heteromer (Feng *et al.*, 2013; Jonas *et al.*, 2015). In contrast, all data were similarly distributed when represented by hCG content of the preparations (Figure 2D; $p\geq0.05$), indicating that FSH content *per se* of Menopur® and Meriofert® may not influence the potency of hCG (and LH) in activating cAMP, in FSHR/LHCGR co-expressing cells. Taken together, these results are indicative of preparation-specific activation of cAMP, detectable in cells expressing FSHR, while these differences are smoothed in FSHR/LHCGR-coexpressing cells.

Analysis of the impact of gonadotropin hormone preparations on intracellular Ca^{2+} release showed inconsistent results (Fig S2; supplementary material) which is overall activated only by the reference gonadotropins at supraphysiological doses or, at least, representative of the gonadotropin concentration at the LH surge.

FSHR- and LHCGR-mediated β-arrestin 2 recruitment

FSHR-dependent recruitment of β-arrestins is important for FSH-dependent ERK-MAPK signaling.

We therefore utilized BRET to investigate the concentration-dependent effects of gonadotropin preparations on β-arrestin 2 recruitment in HEK293 cells expessing FSHR (Figure 3A), LHCGR (Figure 3B), or both the receptors (Figure 3C, D), co-transfected with the yPET-tagged β-arrestin 2 biosensor. In FSHR-expressing cells, Menopur® and Meriofert® showed similar β-arrestin 2 recruitment patterns (Figure 3A, Table 3; $p \geq 0.05$), although the two preparations had a lower potency than the reference recombinant FSH Gonal-F®, as indicated by the EC_{50} values ($p<0.05$). Similar results were obtained in LHCGR-expressing cells (Figure 3B, Table 3), where Menopur®, Meriofert® and the reference hCG control preparation all induced similar β-arrestin 2 recruitment (Figure 3B).

In FSHR and LHCGR co-expressing HEK293 cells, there were no preparation-specific effects on β-arrestin 2 recruitment when data were represented by FSH content (Table 3; $p \geq 0.05$). However, when represented by hCG amount, Meriofert® had a higher potency than Menopur® and Ovitrelle® in inducing the recruitment of β-arrestin 2 (Table 3; Figure 3D; $p<0.05$). Since β-arrestin 2 mediates receptor desensitization after ligand binding (Casarini *et al.*, 2016b; Reiter *et al.*, 2017), these data are suggestive of potential preparation-specific modulatory effects on steroidogenesis.

In cells co-expressing FSHR/LHCGR, cell treatment with recombinant FSH was unable to induce β-arrestin 2 recruitment, indicated by the lack of BRET signal increase with increasing gonadotropin concentration (Figure 3C). These data were confirmed using artificial mixtures of PBS/recombinant FSH (Gonal-F®), hCG (Ovitrelle®) and LH (Luveris®) prepared to reproduce the amount of gonadotropins embedded in Menopur® and Meriofert® preparations (Fig S3), excluding the lack of BRET signal due to the presence of *non*-gonadotropic, perturbing molecules in the commericial batches.

Analysis of gonadotropin-induced β-arrestin 2 recruitment using untagged receptors

β-arrestins are involved in both FSHR and LHCGR desensitization and recycling, and mediate the

activation of signaling modules in time and space, at the intracellular level (Reiter *et al.*, 2017; De Pascali and Reiter, 2018). Therefore, these molecules may play a relevant role in inhibiting ovarian cell death and upregulating granulosa cell proliferation and steroid synthesis (Casarini *et al.*, 2016b). As both LHR and FSHR were tagged with Rluc8 BRET donor we hypothesized that the receptor- β-arrestin 2 BRET signal was due to steric hinderance resulting from the tagged receptors and tridimensional rearrangements incompatible with inducing a BRET signal, rather than the lack of arrestin recruitment to the FSHR-LHCGR heteromer. To evaluate this hypothesis, experiments were repeated, with either FSHR- or LHCGR-Rluc8 tagged and the other receptor untagged. Under the condition of untagged FSHR co-expressed with Rluc8-tagged LHCGR (Figure 4A, B), treatment with FSH was confirmed to fail in inducing a β-arrestin 2 signal (as expected), however BRET was observed in Menopur®- and Meriofert®- treated cells, showing that hCG could induce b-arrestin 2 recruiment. (Table 4; $p \geq 0.05$). In Rluc8-tagged FSHR- and untagged LHCGR-expressing HEK293 cells, treatment with recombinant FSH (Gonal-F®) successfully resulted in a concentration-dependent increase in BRET signal demonstrating β-arrestin 2 recruitment. Interestingly, Gonal-F® displayed a higher potency for recruiting β-arrestin 2 than Menopur®, when drugs are expressed based on FSH content (Figure 4C; Table 4; $p<0.05$). As expected, recombinant hCG (Ovitrelle®) was unable to induce β-arrestin 2 recruitment in Rluc8-FSHR/untagged LHCGR co-expressing cells (Figure 4D; table 4). Both Meriofert and Menopur induced β-arrestin 2 recruitment, moreover, Meriofert® was more potent than Menopur® (Figure 4D; Table 4; $p<0.05$). Therefore, the hCG/LH content of Menopur® may modulate the receptor-receptor tridimensional conformation, while this effect is not mediated by the hCG content of Meriofert®.

Evaluation of progesterone and estradiol production

To understand the physiological impact of the intracellular signaling differences observed with the different gonadotropin hormone preparations, we assessed progesterone and estradiol production in

hGLC with endogenous LHCGR and FSHR expression. For estradiol measurements, androstenedione was added in cell media for providing the aromatase substrate to be converted to the estrogen (Nordhoff *et al.*, 2011). Steroids were quantified by immunoassay and data obtained after 8-h (Figure 5) and 24-h treatments (Figure 6) were plotted in x-y graphs as ng/ml of FSH and hCG, and interpolated by *non*-linear regression. Data plotted considering the total gonadotropin content are also provided (Fig S4),

Gonadotropin exposure for 8 h with data plotted with respect to FSH content revealed no difference in the EC_{50}s in Menopur®, Meriofert® or Gonal-F® (Figure 5; Table 5; $p \geq 0.05$), although there was a trend for decreased potency of Meriofert® in stimulating progesterone production. Interestingly, analysis of data expressed as hCG amount revealed higher potency of Menopur® than Meriofert® in inducing both progesterone and estradiol (Figure 5; Table 5; $p<0.05$), suggesting that lower FSH:hCG ratio and/or the presence of LH (Table 1) may be beneficial for enhancing the steroidogenesis.

Although the evaluation of 24-h progesterone synthesis revealed no differences between the EC_{50} values, when data were plotted to the FSH content (Figure 6; Table 5), differences were observed with respect to hCG content. Menopur® was more potent than Meriofert® in inducing progesterone and estradiol production ($p<0.05$), suggesting that the steroidogenic capability of these preparations is linked to their hormonal composition.

Discussion

This study compared the composition and *in vitro* activities of two hMG preparations Menopur® and Meriofert®, using recombinant hCG (Ovitrelle®) and FSH (Gonal-F®) as references, in FSHR- and/or LHCGR-expressing cells. These drugs are central to ART hormonal regimens. They are utilized to promote multi-follicular development (Casarini *et al.*, 2016a) although differing in hormone composition, glycosylation pattern and origin. Whilst both hMG preparations contain FSH, Menopur® contains both pituitary-derived hCG and LH contrasting to hCG of trophoblastic origin of Meriofert®. The reference drugs, Ovitrelle® and Gonal-F® are of recombinant origin, produced by CHO cells and purified from media. These subtle but key differences form the basis of the *in vitro* drug-specific activities that we described.

Urinary gonadotropin preparations are expressed in IU, as a measure of the *in vivo* bioactivity of an international standard molecule in rodents, determined by *in vivo* bioassays (Steelman and Pohley, 1953; Bangham and Grab, 1964). Since hormone quantification in IU *in vivo* is dependent on several factors such as molecule half-life and clearance (Storring *et al.*, 1981, 1982) in organisms expressing rodent gonadotrophin hormone receptors, which display different binding and signaling profiles (Riccetti *et al.*, 2017c; Nguyen *et al.*, 2018), they are not appropriate for *in vitro* comparisons which utilize human receptors and should be based on the molarity. Therefore, FSH and hCG content of the commercial preparations was re-calibrated by immunometric assay against the reference recombinant gonadotropins, assuming the same immunoreactivity of the different preparations and allowing the number of molecules to be extrapolated (Riccetti *et al.*, 2017a, 2019). We therefore confirmed the FSH and hCG quantities in the hMG formulations, similarly to what was characterized previously (Riccetti *et al.*, 2017b), assuming not significant batch-to-batch variability (Lunenfeld, 2002).

We observed that both hMG preparations had no effect on FSHR-LHCGR heteromeric assembly,

contrasting with the individual FSH, LH and hCG preparations tested. The nature of this effect is likely due to the presence of both ligands. It is plausible that both FSHR and LHCGR binding to their ligand results in the spatial re-arrangement of LHCGR/FSHR heteromers that has no/little effect on the proximity of the BRET tags, therefore causes mimimal effect on the basal BRET signal. This is consistent with data obtained using mono-gonadotropic preparations, i.e. Ovitrelle® and Gonal-F®, which induced an increase in BRET signal, indicating modified ligand induced receptor-receptor interactions. Therefore, we could speculate that the conformation of hCG and FSH-bound heteromers may be different to heteromers with either FSH or hCG bound. On the other hand, FSH, hCG and LH have different steric hindrance (Casarini *et al.*, 2018a) plausibly modulating the receptor spatial organization (Jonas *et al.*, 2015) and specifically impacting G protein activation and downstream signaling (Jonas *et al.*, 2018).

Cell treatments with Menopur® and Meriofert® did not reveal any differences in preparation-specific cAMP activation in cells expressing FSHR or LHCGR alone, nor in cells co-expressing FSHR/LHCGR with respect to FSH content. However, both hMGs were less potent with respect to hCG when normalized for hCG content. These data suggest that receptor heteromers modulate cAMP activation (Jonas et al, 2018; Feng *et al.*, 2013) but this regulatory signaling system may be perturbed by the relative amount of FSH, hCG and LH. In fact, our previous study demonstrated that, in hGLC, nanomolar concentrations of FSH potentiated the effect of picomolar amounts of hCG on cAMP production (Casarini *et al.*, 2016c). Our current data show a similar effect, even if Menopur® and Meriofert® have quite different EC_{50}s than the mixtures of FSH and hCG previously described. This might be due to the amount of FSH and hCG present in the two menotropins, which is different to those used in the previous study (Casarini *et al.*, 2016c). Moreover, we could assume that quantity and proportion of FSHR and LHCGR is widely different between hGLC and transfected HEK293 cells, resulting in different coupling to G proteins and β-arrestins, depending

on the expression level of membrane receptors (Tranchant *et al.*, 2011). This issue may be extended also to granulosa cells collected from follicles at the antral stage, which have reasonably different gonadotropin receptor expression than those of hGLC, suggesting that stage-specific gonadotropin-mediated effects may exist and could be worth of further investigatons.

Our study didn't detect any difference in the ability to recruit β-arrestin 2 between the hMG preparations, in cells singly expressing FSHR or LHCGR. In cells co-expressing FSHR and LHCGR, Menopur® and Meriofert® had a similar potency in recruiting β-arrestin 2, when data were plotted to the FSH content. However, the potency of Meriofert® in recruiting β-arrestin 2 was higher than that of Menopur® and the reference hCG®, when plotted for the hCG amount. These data potentially reflect the contribution of FSH in the action of Meriofert® and Menopur®, which are characterized by different FSH:hCG ratios, with Menopur® containing a lower FSH:hCG, in comparison to Meriofert®. Moreover, Menopur® additionally contains LH, which is absent in Meriofert®, which was previously demonstrated to have lower potency than hCG in inducing β-arrestin 2 recruitment (Riccetti *et al.*, 2017d). We could therefore hypothesize that the preparation-specific composition of hMGs differentially impacts the spatial conformation of active receptors and their homomeric/heteromeric assemblies, as strongly suggested by the BRET biosensor data comparing tagged and untagged receptors. Finally, it is worth noting that EC_{50}s obtained for cAMP activation mismatch those required for β-arrestin 2 recruitment, in FSHR/LHCGR co-expressing cells, which is indicative of potential biased signaling or functional asymmetry within the heteromer, in line with previous reports (Steelman and Pohley, 1953; Storring *et al.*, 1981; Feng *et al.*, 2013; Jonas *et al.*, 2015).

Steroid hormone synthesis is dependent on a number of intracellular factors included cAMP (Chaudhary and Stocco, 1988; Casarini and Crépieux, 2019) and β-arrestins (Ayoub *et al.*, 2016). Therefore, short- and long-term production of both progesterone and estradiol were measured in the

media of steroidogenic cells. Menopur® appeared to be the most potent preparation, together with the reference hCG, in inducing both progesterone and estradiol synthesis, while Meriofert® had a lower potency. These data strengthen the link between steroidogenic pathway and β-arrestins (Ayoub *et al.*, 2016), which would have an inhibitory role on the production of progesterone, but they could also reflect the lower FSH:hCG ratios of Menopur® than Meriofert®, which would result in different steroidogenic efficiency between the two menotropins.

Taken together, this study reveals that the composition of hMGs is linked to specific steroidogenic cell responses, suggesting that they potentially may impact clinical outcomes. Therefore, we provided the mechanistic support to previous researches comparing Meriofert® and Menopur® in a clinical context. A study found that Meriofert® and Menopur® results in similar overall clinical outcomes, except for the shorter duration of the treatment using Meriofert® (Alviggi *et al.*, 2013). A subsequent clinical trial performed using a larger dataset confirmed these results demonstrating the overall clinical equivalence of Menopur® and Meriofert® (Lockwood *et al.*, 2017). Although these results are not conclusive and require to be confirmed by independent studies, *in vitro* data suggest that different FSH:hCG ratio and the presence of LH in hMGs may have a clinical impact (Santi *et al.*, 2017b).

Conclusions

hMG preparations are unique mixture of FSH, hCG (and LH) impacting the receptor heteromeric conformations and resulting in unique downstream signaling signatures. They have a remarkable preparation-specific effect in modulating the synthesis of progesterone and estradiol, suggesting plausible clinical impact. While the clinical studies performed so far overall did not find hMG-specific differences in outcomes and safety, *in vitro* data should be transposed to the clinics cautiously and corroborated by *in vivo* data.

Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author.

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Author's roles

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Authors have no conflict of interest.

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Figure legends

Figure 1. FSHR-LHCGR heteromer modulation by gonadotropin preparations. HEK293 cells were transiently transfected with 50 ng/well of FSH receptor (FSHR)/*Renilla reniformis* luciferase variant 8 (Rluc8) and increasing concentration of LH and hCG receptor (LHCGR)/Venus. Cells were stimulated with gonadotropins for 30 mins, administered as 20 nM FSH content, corresponding to 700 ng/ml (except the reference hCG, which was administered as equal nanomolar amount of this hormone, i.e. 700 ng/ml Ovitrelle®) and bioluminescence resonance energy transfer (BRET) signal recorded at the end of the stimulation period. Data were interpolated by logarithmic function and plotted as means ± standard error of mean (SEM). (**A**) Binding curves indicating FSHR-LHCGR heteromers in transfected cells treated by drugs (r^2 Menopur®=0.71±0.11, n=10; Meriofert®=0.75±0.03, n=7; Gonal-F® recombinant FSH (r-FSH)=0.63±0.13, n=8; Ovitrelle® recombinant hCG (r-hCG)=0.74±0.09, n=6; constitutive=0.70±0.13, n=11). (**B**) Control experiment performed by transfecting HEK293 cells with the FSHR/Rluc8 and vector encoding venus alone. Data were interpolated by linear regression demonstrating non-specific interaction (r^2=0.92; $p<0.0001$; n=4). (**C**, **D**) Control experiments using cells transfected with LHCGR/Rluc8 and Venus (C; r^2 Ovitrelle® (r-hCG)=0.87±0.02; constitutive=0.89±0.01; n=4) or FSHR/Rluc8 and Venus-encoding plasmids (D; r^2 Gonal-F® (r-FSH)=0.63±0.08, n=6; constitutive=0.64±0.07, n=5), treated with the reference hormones, hCG and FSH, respectively. Data analysis was performed by Kruskal Wallis test and Dunn's post-test ($p<0.05$).

Figure 2. Effect of hMGs and recombinant gonadotropin hormone preparations on cAMP

generation. HEK293 cells transiently expressing the CAMYEL sensor were treated with increasing concentrations of hMGs, or recombinant hCG and FSH for 30-mins and cAMP accumulation was measured by BRET. Data were interpolated by nonlinear regression and plotted relative to the amount of FSH or hCG (x-axis). EC_{50} values were compared to that of Menopur® by Kruskall-Wallis test and Dunn's post-test (Table 2; $p<0.05$; n=8; means±SEM). (**A**) effect of gonadotropin hormones on intracellular cAMP in FSHR-expressing HEK293 cells, plotting with respect to the FSH content of each hormone preparation. (**B**) Effect of gonadotropin hormones on intracellular cAMP in LHCGR-expressing HEK293 cells, plotting with respect to the hCG content of each hormone preparation. cAMP responses in FSHR and LHCGR co-expressing cells, with data plotted with respect to (**C**) FSH and (**D**) hCG content.

Figure 3. Comparison of Menopur®- and Meriofert®-induced β-arrestin 2 recruitment. HEK293 cells were transfected with the aequorin sensor-encoding plasmid, ± individual or both FSHR/Rluc8 and LHCGR/Rluc8. Cells were treated for 30 min and β-arrestin 2 recruitment was evaluated by BRET. Data interpolation was performed by nonlinear regression and the comparison between Menopur® EC_{50} *versus* those of other drugs were performed using Kruskall-Wallis test and Dunn's post-test. Data were plotted based on the amount of FSH or hCG on each preparation. (Table 3; $p<0.05$; n=8; means±SEM). (**A**) β-arrestin 2 recruitment in FSHR/Rluc8-expressing HEK293 cells, with data plotted on the x-axis based on the FSH content of the gonadotropin hormone preparation. (**B**) β-arrestin 2 recruitment in LHCGR/Rluc8-expressing HEK293 cells, with data plotted on the x-axis based on the hCG content of the gonadotropin hormone preparation. β-arrestin 2 recruitment in FSHR and LHCGR co-expressing cells, with data plotted based on **(C)** FSH or **(D)** hCG content.

Figure 4. Assessment of drug-dependent FSHR/LHCGR/β-arrestin 2 conformational rearrangement. HEK293 cells were transfected with the aequorin sensor-encoding plasmid and treated with gonadotropin hormone preparations for 30 mins. Following this, β-arrestin 2 recruitment was evaluated by BRET and nonlinear regression used for interpolating data with the Menopur® EC_{50} was compared to others using the Kruskall-Wallis test and Dunn's post-test (Table 4; $p<0.05$; n=8; means±SEM). Data were were plotted with respect to the amount of FSH or hCG in each preparation. β-arrestin 2 recruitment in HEK293 cells expressing untagged FSHR and LHCGR/Renilla reniformis luciferase variant 8 (Rluc8), plotting considering the (**A**) FSH or (**B**) hCG content. β-arrestin 2 recruitment in cells co-expressing the FSHR/Rluc8 biosensor and the untagged LHCGR, plotting for (**C**) FSH or (**D**) hCG content.

Figure 5. The effects of acute Menopur®- and Meriofert® treatment on steroid hormone production in hGLC. hGLC were treated for 8 hrs with gonadotropin hormone preparations, and progesterone and estradiol were measured by immunoassay. Values were interpolated by nonlinear regression plotted considering the FSH or hCG content. EC_{50} values were compared to that of Menopur® by Kruskall-Wallis test and Dunn's post-test (Table 5; $p<0.05$; n=8; means±SEM). 8-hr progesterone synthesis plotted with respect to the **(A)** FSH or **(B)** hCG content. 8-hr estradiol levels plotted against **(C)** FSH or **(D)** hCG content.

Figure 6. The effects of chronic Menopur®- and Meriofert® treatment on steroid hormone production in hGLC. hGLCs were treated for 24 hrs with commercial gonadotropin hormone preparations. Progesterone and estradiol were measured by immunoassay, and data interpolatied by nonlinear regression and plotted (means ± SEM) with respect to the FSH and hCG content. The Menopur® EC_{50} value was compared to others (Table 5; Kruskall-Wallis test and Dunn's post-test;

p<0.05; n=8). 24-hr progesterone levels plotted for **(A)** FSH and **(B)** hCG content. 24-hr estradiol levels plotted considering the (C) FSH and (D) hCG content.

Supplementary figure legends

Fig S1. Control Western blotting for FSHR, LHCGR and β-arrestin 2 overexpression. HEK293 were transiently co-transfected by FLAG-tagged FSHR- and LHCGR-, and β-arrestin 2-encoding plasmids, and target molecules were detected using specific primary antibody. Cells transfected by the empty pcDNA3.1 vector (mock-transfected) were used as a negative control. A) Treatment by the anti-FSHR antibody of membranes loaded by FSHR/LHCGR/β-arrestin 2 overexpressing HEK293 and mock-transfected cell lysates. B) anti-LHCGR antibody. C) Anti-FLAG antibody recognizing both the FLAG-tagged FSHR and LHCGR. D) Treatment by the anti-β-arrestin 2 antibody of membranes loaded by 20-fold lower cell lysates than those used for panels A-C for avoiding endogenous β-arrestin 2 detection.

Fig S2. Drug-induced kinetics of intracellular Ca^{2+} increase, in FSHR/LHCGR co-expressing HEK293 cells. Cell treatments were performed by injecting the drug at the 50 s time-point, while GFP/aequorin biosensor light emissions were detected continuously over 250 s. hCG content of Meriofert® was not enough for achieving the Ca^{2+}-minimally increasing concentration of 2 µg/ml compared to the negative control (Kruskal-Wallis test; p≥0.05; n=3; means±SEM). A) 2 µg/ml Ovitrelle® (r-hCG)-induced intracellular Ca^{2+} increase. B) Kinetics of Ca^{2+} increase induced by Menopur® administered for achieving 2 µg/ml of hCG content. C) 1.7 µg/ml Gonal-F® (r-FSH)-induced intracellular Ca^{2+} increase. D) Menopur® administered for achieving 1.7 µg/ml of FSH content. E) Meriofert® administered for achieving 1.7 µg/ml of FSH content. E) Negative control (PBS). F) positive control (thapsigargin).

Fig S3. β arrestin-2 recruitment induced by artificial mixtures of gonadotropins reproducing Menopur® and Meriofert®, in HEK293 cells transiently co-expressing FSHR LHCGR and β-arrestin 2 BRET biosensors. Cells were treated by increasing drug concentrations and β arrestin-2 recruitment was measured by BRET. Artificial drugs were assembled as follows: Meriofert® = 8.00 μM r-FSH, 120.00 nM r-hCG; Menopur® = 80.00 μM r-FSH, 2.95 μM r-hCG, 320.00 nM r-LH. A) Results plotted for FSH content at the x-axis, except the Ovitrelle® preparation (r-hCG), which was plotted considering the amount of hCG. B) hCG content at the x-axis, except the Gonal-F® preparation (r-hFSH), which was plotted considering the amount of FSH.

Fig S4. 8- and 24-h progesterone and estradiol production plotted considering the total amount of gonadotropins. Cells were treated with commercial drugs and steroid levels measured in the cell media after 8 and 24 h, by immunoassay. Data were interpolated by nonlinear regression (means±SEM n=8) considering the amount of FSH or hCG at the x-axis (except for the Ovitrelle® and Gonal-F® preparations, which were plotted considering the amount of hCG and FSH, respectively, at the x-axis). A) 8-h progesterone production. B) 8-h estradiol production. C) 24-h progesterone production. D) 24-h estradiol production.

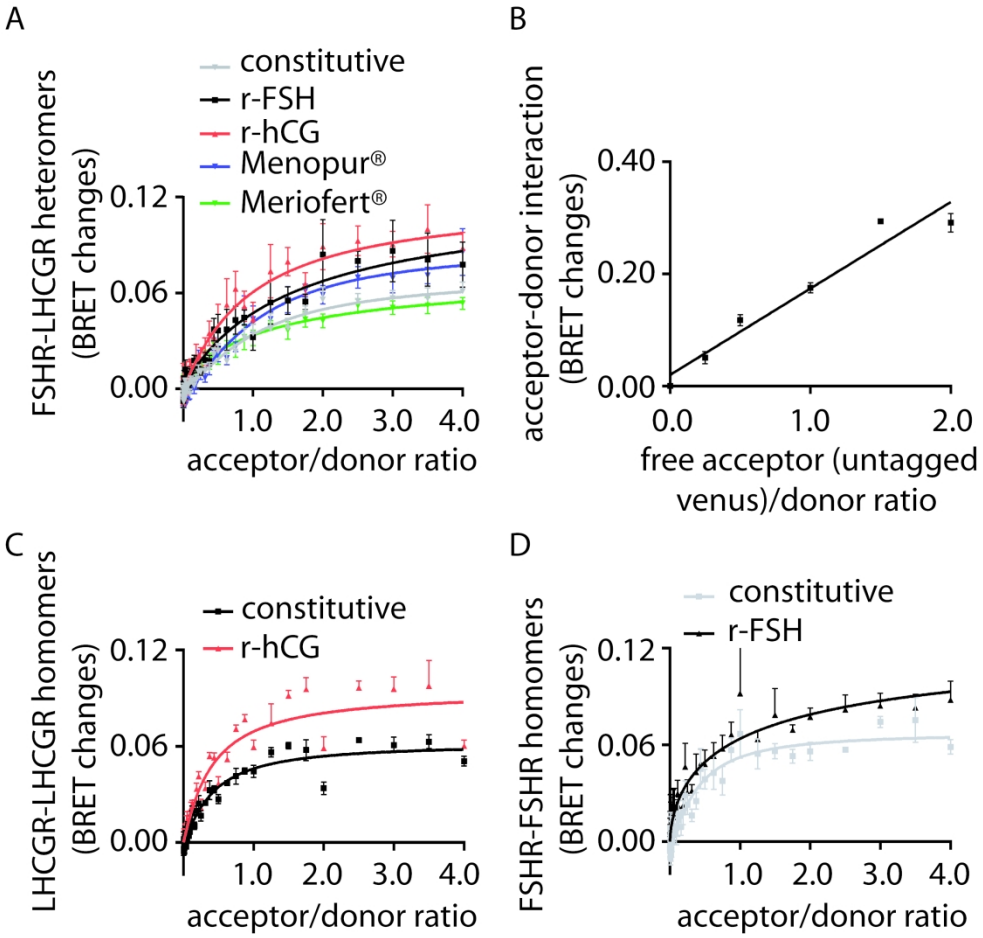

Figure 1. FSHR-LHCGR heteromer modulation by gonadotropin preparations. HEK293 cells were transiently transfected with 50 ng/well of FSHR/Rluc8 and increasing concentration of LHCGR/Venus. Cells were stimulated with gonadotropins for 30 mins, administered as 20 nM FSH content, corresponding to 700 ng/ml (except the reference hCG, which was administered as equal nanomolar amount of this hormone, i.e. 700 ng/ml Ovitrelle®) and BRET signal recorded at the end of the stimulation period. Data were interpolated by logarithmic function and plotted as means ± SEM. (A) Binding curves indicating FSHR-LHCGR heteromers in transfected cells treated by drugs (r^2 Menopur®=0.71±0.11, n=10; Meriofert®=0.75±0.03, n=7; Gonal-F® (r-FSH)=0.63±0.13, n=8; Ovitrelle® (r-hCG)=0.74±0.09, n=6; constitutive=0.70±0.13, n=11). (B) Control experiment performed by transfecting HEK293 cells with the FSHR/Rluc8 and vector encoding venus alone. Data were interpolated by linear regression demonstrating non-specific interaction (r^2=0.92; p<0.0001; n=4). (C, D) Control experiments using cells transfected with LHCGR/Rluc8 and Venus (C; r^2 Ovitrelle® (r-hCG)=0.87±0.02; constitutive=0.89±0.01; n=4) or FSHR/Rluc8 and Venus-encoding plasmids (D; r^2 Gonal-F® (r-FSH)=0.63±0.08, n=6; constitutive=0.64±0.07, n=5), treated with the reference hormones, hCG and FSH, respectively. Data analysis was performed by Kruskal Wallis test and Dunn's post-test (p<0.05).

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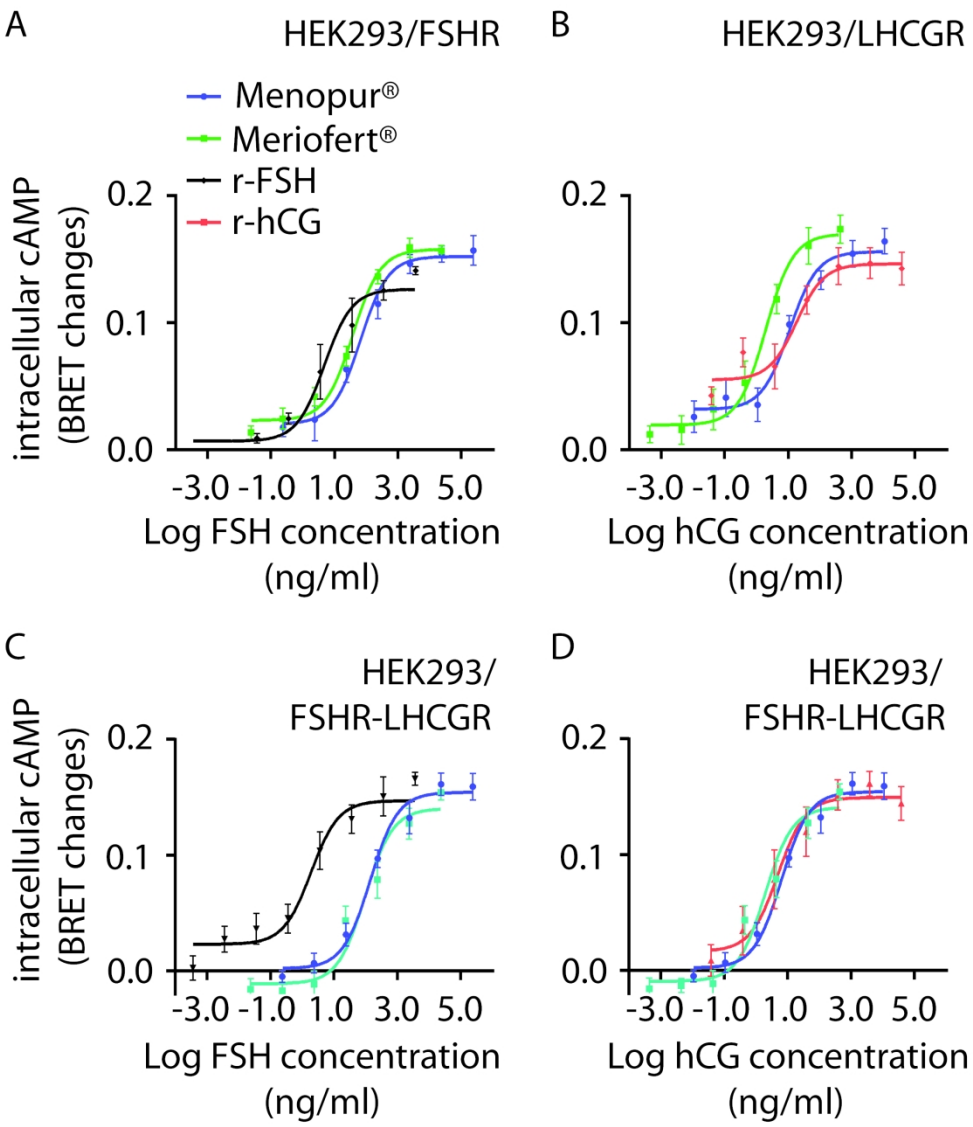

Figure 2. Effect of hMGs and recombinant gonadotropin hormone preparations on cAMP generation. HEK293 cells transiently expressing the CAMYEL sensor were treated with increasing concentrations of hMGs, or recombinant hCG and FSH for 30-mins and cAMP accumulation was measured by BRET. Data were interpolated by nonlinear regression and plotted relative to the amount of FSH or hCG (x-axis). EC_{50} values were compared to that of Menopur® by Kruskall-Wallis test and Dunn's post-test (Table 2; $p<0.05$; n=8; means±SEM). (A) effect of gonadotropin hormones on intracellular cAMP in FSHR-expressing HEK293 cells, plotting with respect to the FSH content of each hormone preparation. (B) Effect of gonadotropin hormones on intracellular cAMP in LHCGR-expressing HEK293 cells, plotting with respect to the hCG content of each hormone preparation. cAMP responses in FSHR and LHCGR co-expressing cells, with data plotted with respect to (C) FSH and (D) hCG content.

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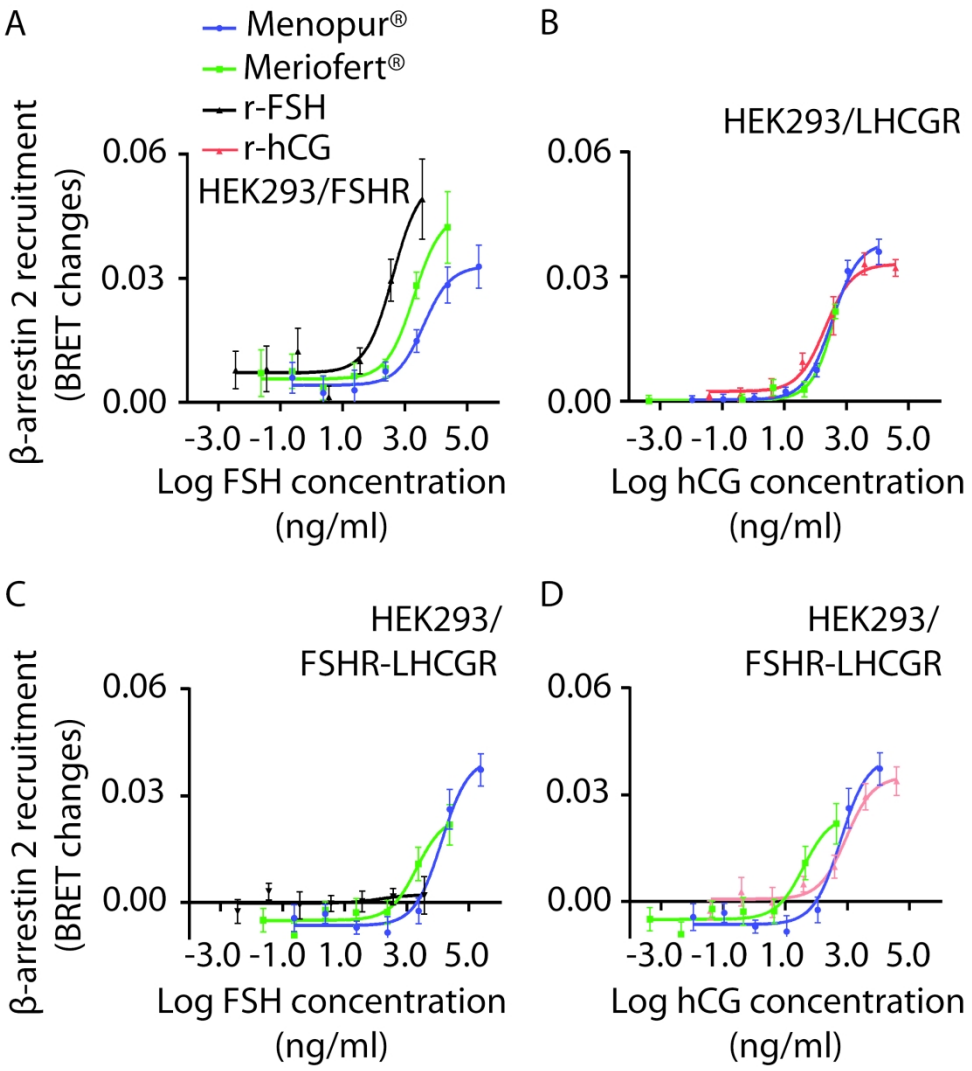

Figure 3. Comparison of Menopur®- and Meriofert®-induced β-arrestin 2 recruitment. HEK293 cells were transfected with the aequorin sensor-encoding plasmid, ± individual or both FSHR/Rluc8 and LHCGR/Rluc8. Cells were treated for 30 min and β-arrestin 2 recruitment was evaluated by BRET. Data interpolation was performed by nonlinear regression and the comparison between Menopur® EC_{50} *versus* those of other drugs were performed using Kruskall-Wallis test and Dunn's post-test. Data were plotted based on the amount of FSH or hCG on each preparation. (Table 3; $p<0.05$; n=8; means±SEM). (A) β-arrestin 2 recruitment in FSHR/Rluc8-expressing HEK293 cells, with data plotted on the x-axis based on the FSH content of the gonadotropin hormone preparation. (B) β-arrestin 2 recruitment in LHCGR/Rluc8-expressing HEK293 cells, with data plotted on the x-axis based on the hCG content of the gonadotropin hormone preparation. β-arrestin 2 recruitment in FSHR and LHCGR co-expressing cells, with data plotted based on (C) FSH or (D) hCG content.

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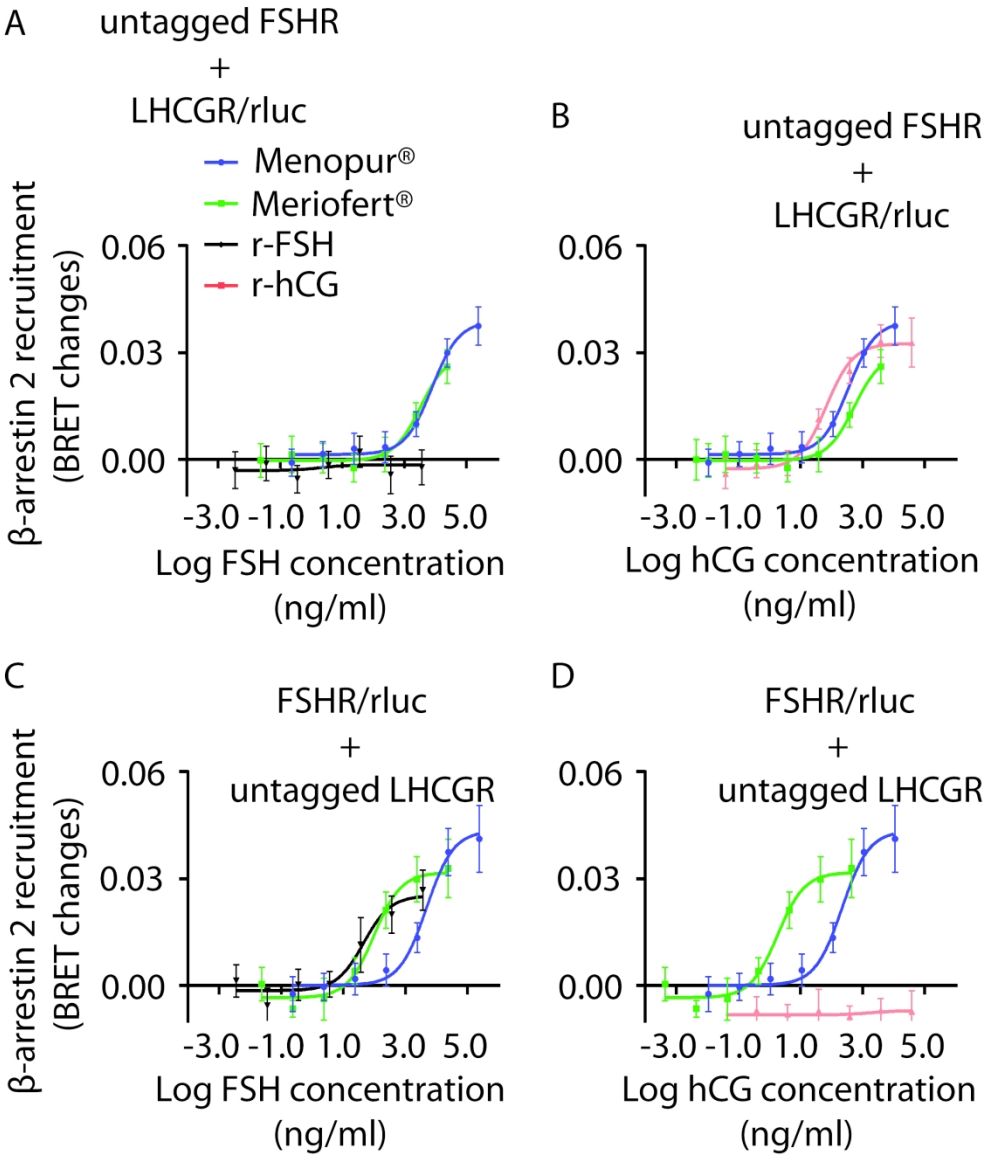

Figure 4. Assessment of drug-dependent FSHR/LHCGR/β-arrestin 2 conformational rearrangement. HEK293 cells were transfected with the aequorin sensor-encoding plasmid and treated with gonadotropin hormone preparations for 30 mins. Following this, β-arrestin 2 recruitment was evaluated by BRET and nonlinear regression used for interpolating data with the Menopur® EC_{50} was compared to others using the Kruskall-Wallis test and Dunn's post-test (Table 4; $p<0.05$; n=8; means±SEM). Data were were plotted with respect to the amount of FSH or hCG in each preparation. β-arrestin 2 recruitment in HEK293 cells expressing untagged FSHR and LHCGR/Rluc8, plotting considering the (A) FSH or (B) hCG content. β-arrestin 2 recruitment in cells co-expressing the FSHR/Rluc8 biosensor and the untagged LHCGR, plotting for (C) FSH or (D) hCG content.

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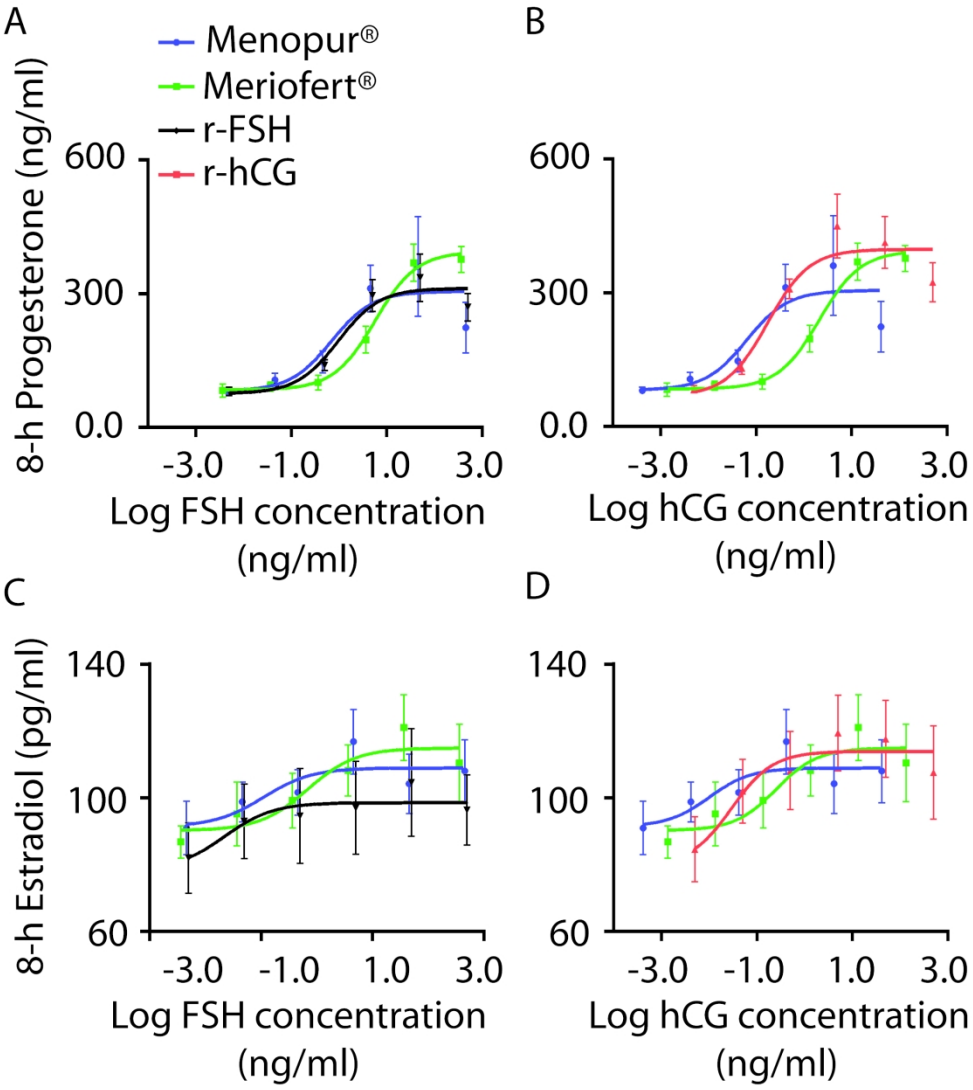

Figure 5. The effects of acute Menopur®- and Meriofert® treatment on steroid hormone production in hGLC. hGLC were treated for 8 hrs with gonadotropin hormone preparations, and progesterone and estradiol were measured by immunoassay. Values were interpolated by nonlinear regression plotted considering the FSH or hCG content. EC_{50} values were compared to that of Menopur® by Kruskall-Wallis test and Dunn's post-test (Table 5; $p<0.05$; n=8; means±SEM). 8-h progesterone synthesis plotted with respect to the (A) FSH or (B) hCG content. 8-h estradiol levels plotted against (C) FSH or (D) hCG content.

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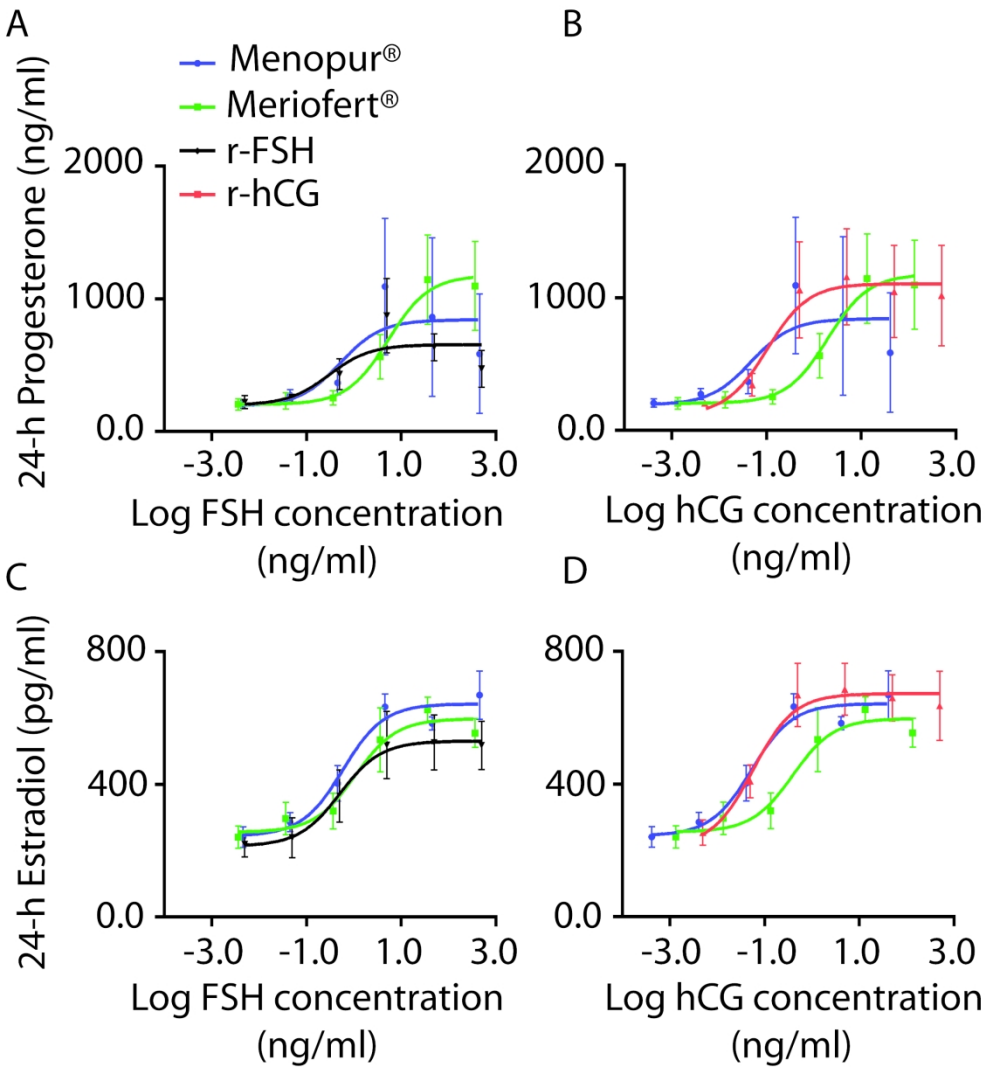

Figure 6. The effects of chronic Menopur®- and Meriofert® treatment on steroid hormone production in hGLC. hGLCs were treated for 24 h with commercial gonadotropin hormone preparations. Progesterone and estradiol were measured by immunoassay, and data interpolatied by nonlinear regression and plotted (means±SEM) with respect to the FSH and hCG content. The Menopur® EC_{50} value was compared to others (Table 5; Kruskall-Wallis test and Dunn's post-test; $p<0.05$; $n=8$). 24-h progesterone levels plotted for (A) FSH and (B) hCG content. 24-h estradiol levels plotted considering the (C) FSH and (D) hCG content.

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Table 1. FSH, hCG and LH immunoreactivity and quantification of commercial preparations

Preparation	Activity	Mass		FSH content measured		hCG content measured		LH content
	IU-batch declared	ng-batch declared	mIU-batch/ml loaded	Immunoreactivity (mIU/ml)	Quantification (ng/ml)	Immunoreactivity (mIU/ml)	Quantification (ng/ml)	Immunoreactivity (mIU/ml)
Menopur®	75	Not available	5000	3919.4 ± 11.8	287.4 ± 0.9	677.6 ± 17.3	26.1 ± 0.5	26.9 ± 0.5
Meriofert®	75	Not available	5000	3893.3 ± 6.5	285.6 ± 0.5	273.4 ± 11.9	10.5 ± 0.5	Not det
Gonal-f®	1050	77000	5000	1166.7 ± 2.3	85.6 ± 0.2	Not detectable		Not det
Ovitrelle®	6500	250000	5000	Not detectable		9544.0 ± 103.2	367.1 ± 3.9	Not det
Luveris®	75	3400	5000	Not detectable		Not detectable		1550.5 ± 60.2

Data are indicated as the mean ± standard error of mean (SEM).

: **measured**

Quantification (ng/ml)
1.2 ± 0.02
:ectable
:ectable
:ectable
70.3 ± 2.7

Table 2.EC_{50} values of gonadotropin preparations required for cAMP activation.

Preparation	FSHR-expressing HEK293 Representation for FSH content	LHCGR-expressing HEK293 Representation for hCG content	FSHR/LHCGR-exp Representation for FSH content
Menopur®	66.1 ± 49.1	**11.0 ± 12.0**	**140.0 ± 109.6**
Meriofert®	37.9 ± 19.3	**2.8 ± 1.1 ***	97.0 ± 99.3
Gonal-f®	4.8 ± 44.3	not available	**1.9 ± 8.3 ***
Ovitrelle®	not available	18.6 ± 25.26	not available

*significantly different versus Menopur® (Kruskall-Wallis test and Dunn's post-test; p<0.05; n=8; data are n

pressing HEK293	Representation for hCG content
	6.4 ± 9.6
	1.9 ± 18.4
	not available
	2.1 ± 53.1

neans ± SEM and indicated as ng/ml). Abbreviations: FSH receptor (FSHR); LH and hCG receptor (LHCGR).

Table 3.EC_{50} values of gonadotropin preparations measured for β-arrestin 2 recruitment.

Preparation	FSHR-expressing HEK293 Representation for FSH content	LHCGR-expressing HEK293 Representation for hCG content	FSHR/LHCGR-exp Representation for FSH content
Menopur®	**3968.0 ± 287.7**	341.6 ± 138.0	13080.0 ± 4715.0
Meriofert®	1960.0 ± 398.6	942.2 ± 267.2	2063 ± 1292.3
Gonal-f®	**413.6 ± 46.9 ***	not available	undetectable
Ovitrelle®	not available	208.2 ± 130.7	not available

*significantly different versus Menopur® (Kruskall-Wallis test and Dunn's post-test; $p<0.05$; n=8; data are n

pressing HEK293
Representation for hCG content
599.1 ± 147.2
38.3 ± 29.2 *
not available
912.7 ± 234.5

neans ± SEM and indicated as ng/ml)

Table 4.EC_{50} values of gonadotropin preparations measured for β-arrestin 2 recruitment in FSHR/LHCGR-ex

Preparation	untagged FSHR and LHCGR-Rluc8		FSHR-Rluc8 and u
	Representation for FSH content	**Representation for hCG content**	**Representation for FSH content**
Menopur®	7689.0 ± 374.2	352.0 ± 137.4	**4751.0 ± 2433.6**
Meriofert®	3266.0 ± 300.8	533.8 ± 230.1	983.1 ± 851.7
Gonal-f®	undetectable	not available	**447.9 ± 115.9 ***
Ovitrelle®	not available	67.8 ± 135.2	not available

*significantly different versus Menopur® (Kruskall-Wallis test and Dunn's post-test; $p<0.05$; n=8; data are m

xpressing HEK293 cells.

untagged LHCGR
Representation for hCG content
217.5 ± 43.4
1.8 ± 0.1 *
not available
undetectable

neans ± SEM and indicated as ng/ml). Abbreviations: *Renilla reniformis* luciferase variant 8 (Rluc8).

Table 5. Gonadotropin preparation EC_{50} values calculated for progesterone and es

Preparation	Representation for FSH content	Representation for hCG content
	8-h progesterone	
Menopur®	0.70 ± 0.05	**0.06 ± 0.05**
Meriofert®	5.75 ± 0.17	**2.11 ± 0.17 ***
Gonal-f®	0.97 ± 0.03	not available
Ovitrelle®	not available	0.17 ± 0.03
	24-h progesterone	
Menopur®	0.50 ± 0.05	**0.05 ± 0.05**
Meriofert®	5.36 ± 0.20	**1.98 ± 0.20 ***
Gonal-f®	0.30 ± 0.04	not available
Ovitrelle®	not available	0.10 ± 0.03

*significantly different versus Menopur® (Kruskall-Wallis test and Dunn's post-test

stradiol production in hGLC.

Representation for FSH content	Representation for hCG content
8-h estradiol	
0.11 ± 0.01	**0.01 ± 0.01**
0.69 ± 0.08	**0.25 ± 0.08 ***
0.02 ± 0.01	not available
not available	0.03 ± 0.01
24-h estradiol	
0.59 ± 0.23	**0.05 ± 0.23**
1.09 ± 0.36	**0.40 ± 0.36 ***
0.51 ± 0.46	not available
not available	0.06 ± 0.04

t; p<0.05; n=5; data are means ± SEM and indicated as x1000 ng/ml.)