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ORIGINAL ARTICLE

Heterogeneous hCG and hMG commercial preparations result in different intracellular signalling but induce a similar long-term progesterone response *in vitro*

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STUDY QUESTION: Are four urinary hCG/menotropin (hMG) and one recombinant preparation characterized by different molecular features and do they mediate specific intracellular signaling and steroidogenesis?

SUMMARY ANSWER: hCG and hMG preparations have heterogeneous compositions and mediate preparation-specific cell signaling and early steroidogenesis, although similar progesterone plateau levels are achieved in 24 h-treated human primary granulosa cells *in vitro*.

WHAT IS KNOWN ALREADY: hCG is the pregnancy hormone marketed as a drug for ARTs to induce final oocyte maturation and ovulation, and to support FSH action. Several hCG formulations are commercially available, differing in source, purification methods and biochemical composition.

STUDY DESIGN, SIZE, DURATION: Commercial hCG preparations for ART or research purposes were compared *in vitro*.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The different preparations were quantified by immunoassay with calibration against the hCG standard (Fifth IS; NIBSC 07/364). Immunoreactivity patterns, isoelectric points and oligosaccharide contents of hCGs were evaluated using reducing and non-reducing Western blotting, capillary isoelectric-focusing immunoassay and lectin-ELISA, respectively. Functional studies were performed in order to evaluate intracellular and total cAMP, progesterone production and β-arrestin 2 recruitment by ELISA and BRET, in both human primary granulosa lutein cells (hGLC) and luteinizing hormone (LH)/hCG receptor (LHCGR)-transfected HEK293 cells, stimulated by increasing hormone concentrations. Statistical analysis was performed using two-way ANOVA and Bonferroni post-test or Mann–Whitney's *U*-test as appropriate.

MAIN RESULTS AND THE ROLE OF CHANCE: Heterogeneous profiles were found among preparations, revealing specific molecular weight patterns (20–75 KDa range), isoelectric points (4.0–9.0 pI range) and lectin binding ($P < 0.05$; $n = 7$–10). These drug-specific

compositions were linked to different potencies on cAMP production (EC_{50} 1.0–400.0 ng/ml range) and β-arrestin 2 recruitment (EC_{50} 0.03–2.0 μg/ml) in hGLC and transfected HEK293 cells ($P < 0.05$; $n = 3$–5). In hGLC, these differences were reflected by preparation-specific 8-h progesterone production although similar plateau levels of progesterone were acheived by 24-h treatment ($P \geq 0.05$; $n = 3$).

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: The biological activity of commercial hCG/hMG preparations is provided in International Units (IU) by *in-vivo* bioassay and calibration against an International Standard, although it is an unsuitable unit of measure for *in-vitro* studies. The re-calibration against recombinant hCG,quantified in grams, is based on the assumption that all of the isoforms and glycosylation variants have similar immunoreactivity.

WIDER IMPLICATIONS OF THE FINDINGS: hCG/hMG preparation-specific cell responses *in vitro* may be proposed to ART patients affected by peculiar ovarian response, such as that caused by polycystic ovary syndrome. Otherwise, all the preparations available for ART may provide a similar clinical outcome in healthy women.

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Key words: hCGhCG / hMGhMG / FSHFSH / LHLH / gonadotropin

Introduction

hCG is a heterodimeric glycoprotein hormone, supporting pregnancy in primates. hCG is crucial to rescue the corpus luteum from atresia and to maintain progesterone production, thereby ensuring embryo implantation and development (Pierce and Parsons, 1981; Cole, 2012a). The quaternary structure of hCG is enriched by carbohydrate residues covalently bound to the peptidic chains. They consist of four N- and four O-linked oligosaccharides. Sialic acid content is crucial for receptor binding, hCG biological activity and clearance from maternal circulation (de Medeiros and Norman, 2009). hCG exerts its biological functions by binding to the lutropin/choriogonadotropin receptor (LHCGR). LHCGR is a G protein-coupled receptor (GPCR) belonging to the rhodopsin/β2-adrenergic receptor subfamily and its activation triggers both G protein- and β-arrestin-mediated pathways (Legardinier *et al.*, 2005; Casarini *et al.*, 2012; Heitzler *et al.*, 2012; Ayoub *et al.*, 2015, 2016), resulting in downstream steroidogenesis, proliferative, and anti- and pro-apoptotic signals. β-arrestin 2 is involved in desensitization/internalization events and in downstream ERK1/2 signaling pathways activation (Shenoy and Lefkowitz, 2011; Reiter *et al.*, 2012; Thomsen *et al.*, 2016). At least four hCG isoforms are detectable in serum and urine samples, differing in oligosaccharides moieties, half-life and biological activity (Kardana *et al.*, 1991; Butler *et al.*, 2001; Cole, 2012b). Moreover, a hyperglycosylated hCG variant may be secreted by invasive cytotrophoblast cells during the first trimester of pregnancy (Takamatsu *et al.*, 1999), whose mechanism of action is still under discussion (Berndt *et al.*, 2013; Koistinen *et al.*, 2015).

Gonadotropins are largely used for ovarian stimulation in assisted reproduction. In particular, hCG may be used to support FSH-induced multifollicular growth and oocyte trigger, in several clinical protocols (Casarini *et al.*, 2016a). Human menopausal gonadotropin (hMG) was the first preparation used in clinical practice and has been available since the 1950s (Loraine and Brown, 1955). It was originally purified from pooled urine of post-menopausal female donors and contains both FSH and LH molecules. Other urinary gonadotropins have been developed in the last two decades, providing highly-purified hCG preparations suitable for ARTs. Recombinant gonadotropins, obtained from transfected Chinese hamster ovary (CHO) cell lines, are also used (Siebold, 1996). Importantly, all these preparations differ in composition, source and purification methods, suggesting that gonadotropin-specific effects may occur (Casarini *et al.*, 2016a).

The high degree of purification of gonadotropins was demonstrated by previous studies comparing recombinant vs urinary gonadotropins, which revealed no variability in ART performances between these preparations (Youssef *et al.*, 2016). Moreover, high batch-to-batch consistency may be assumed for both recombinant and urinary preparations, as suggested by biochemical analyses and evaluations of clinical outcomes (Gervais *et al.*, 2003; Hugues and Durnerin, 2005; Wolfenson *et al.*, 2005). These reports demonstrated the suitability of gonadotropin preparations for ART, although an in-depth molecular characterization and an analysis of their action *in vitro* has never been published.

Aim of the study

In this study, five urinary and recombinant hCG/hMG preparations were analyzed for biochemical composition and presence of glycosylation isoforms. cAMP production, β-arrestin 2 recruitment and progesterone synthesis were also investigated, in both HEK293 cells transiently expressing LHCGR and human primary granulosa cells naturally expressing LHCGR.

Materials and Methods

Gonadotropin commercial preparations

Four different hCG and one hMG preparations were analyzed: Pregnyl® 5000 IU (Organon International, Oss, Holland), Gonasi HP® 5000 IU (IBSA Farmaceutici Italia, Pambio-Noranco, Switzerland), hCG from pregnancy urine for research purpose C0434 50 μg (Sigma-Aldrich, St. Louis, MO, USA), Menopur® 75 IU (Lot. N. CE0566A; a gift from Ferring Pharmaceuticals, Saint Prex, Switzerland) and recombinant hCG (rhCG) Ovitrelle® 250 μg (Merck KGaA, Darmstadt, Germany). Recombinant FSH (rFSH) Gonal-F® 75 IU and recombinant LH (rLH) Luveris® 75 IU (both from Merck KGaA), or natural highly-purified pituitary human LH or FSH (hLH-SIAFP1, 4500 IU/mg; hFSH-SIAFP1, 4500 IU/mg) both provided by Dr A. F. Parlow (NHPP NIDDK, Torrance, CA, USA), served as controls. As declared by the Supplier, 75 IU of Menopur® *in-vivo* activity results from 75 IU FSH and 75 IU LH, where lutropin activity is provided mainly by pituitary hCG (van de Weijer *et al.*, 2003), although Menopur® even contains a relatively low amount of LH.

Cell culture and transfection

Both primary cells and transiently transfected cell lines were used. Human primary granulosa lutein cells (hGLC) were isolated from ovarian follicles of women undergoing oocyte retrieval for ART. Patients had to match the following criteria: absence of endocrine abnormalities and viral/bacterial infections, age between 25 and 45 years.

Cells were recovered using a 50% Percoll density gradient (GE Healthcare, Little Chalfont, UK), following a validated protocol (Casarini *et al.*, 2012, 2014, 2016b). hGLC from three to four patients were pooled and cultured 5–6 days before experiments to recover LHCGR expression (Nordhoff *et al.*, 2011). Cells were cultured at 37°C and 5% CO_2 in McCoy's 5 A medium, supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml Fungizone (all from Sigma-Aldrich). HEK293 cells were grown in complete DMEM medium supplemented with 10% FBS, 4.5 g/l glucose, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 1 mM glutamine (Invitrogen, Carlsbad, CA, USA). Transient transfections were performed in 96-well plate using Metafectene PRO (Biontex Laboratories GmbH, München, Germany), following the manufacturer's instructions. Briefly, 100 ng/well plasmid and 0.5 µl/well of Metafectene PRO were mixed in serum-free medium and incubated 20 min. A 50 µl plasmid-Metafectene PRO mix was added to each wells containing 1×10^5 cells, in a total volume of 200 µl/well, and incubated 2 days before stimulation by gonadotropins. All of the preparations were tested together in each experimental replicate to avoid results biased by patient-specific cell response or HEK293 cells transfection.

Ethical approval

Study approval from the local Ethic Committee was obtained (No. 796, 19 June 2014, released by Comitato Etico Provinciale, Reggio Emilia, Italy). Donors were anonymous and signed a written consent.

Immunoassay to quantify hCG content

hCG immunoreactivity of commercial preparations was evaluated by immunoenzymatic sandwich assay (#A85264, Access Immunoassay Systems; Beckman Coulter Inc., Brea, CA, USA) calibrated against the hCG standard (Fifth IS; NIBSC 07/364), following the manufacturer's instructions. Briefly, hCG immobilization on paramagnetic particles occurs by binding to a mouse monoclonal anti-hCGβ antibody in complex with a goat anti-mouse IgG. A rabbit anti-hCGβ alkaline phosphatase conjugate is added and reacts with the immobilized hCG, which is retained unlike the unbound materials. Specifically, serial dilutions starting from 5000 mIU/ml of all the samples were loaded within the same assay, following the IU dosage determined by the suppliers (using *in vivo* bioassays). Light emission occurring upon addition of Lumi-Phos* 530 chemiluminescent substrate (Lumigen Inc., Beckman Coulter Inc.) is measured by a DxI 800 luminometer (Beckman Coulter Inc.) and compared against the multi-point calibration curve of the hCG standard (Fifth IS; NIBSC 07/364) (Vankrieken and De Hertogh, 1995). The Supplier provided approximate reactivity to the following hCG preparation as follows: intact hCG (WHO 9/688) ~109%; free hCGβ (WHO 99/650) ~193%; nicked intact hCG (WHO 99/642) ~127%; nicked free hCGβ subunit (WHO 99/692) ~127%; free α-subunits and β-core fragments, not detectable.

FSH and LH were measured using an ARCHITECT chemiluminescent immunoassay system (Abbot Diagnostics; Abbott Park, Chicago, IL, USA), where the calibrators were standardized against first IS WHO 92/510 and second IS WHO80/522, respectively. FSH and LH measurement relied on mouse monoclonal anti-FSHβ and -LHβ antibodies provided in the assay kits (#7K75 and #2P40, respectively; Abbott Diagnostics).

Sds page, silver staining analysis and Western blotting analysis

hCG/hMG preparations were resolved on 12% SDS-PAGE. Briefly, samples were loaded according to gonadotropin quantification obtained by immunoassay (0.05–3.00 µg). Gel electrophoresis was performed under denaturing (by heating)-reducing or non-denaturing-reducing conditions, and silver staining and Western blotting analysis was carried out. Silver staining was performed after acrylamide gel electrophoresis. Gels were fixed 1 h by 50% ethanol buffer, 12% acetic acid, 5×10^{-4}% formalin (all from Sigma-Aldrich). After three washes by 50% ethanol, gels were incubated for 1 min in 0.01% $Na_2S_2O_3$, followed by a 20 min wash in deionized H_2O. Gel staining was performed by 0.2% $AgNO_3$ buffer for 30 min followed by three washes using deionized H_2O. Signals were developed by 1 h incubation with 3% Na_2CO_3 buffer, 0.0005% formalin and 4×10^{-4}% $Na_2S_2O_3$ buffer. Reactions were stopped by washing gels in 50% methanol, 12% acetic acid buffer (Chevallet *et al.*, 2006). hCG isoforms, LH and FSH detection was performed by 12% acrylamide SDS page Western blotting. Rabbit anti-human polyclonal antibody against hCGβ was used to recognize both free hCGβ-subunits and dimeric hCGs (#A0231; Dako, Agilent Technologies, Santa Clara, CA, USA), and a rabbit anti-human polyclonal antibody against FSHβ/FSH (SAB1304978; Sigma-Aldrich). Signals were detected by a ECL chemiluminescent compound (GE HealthCare, Little Chalfont, UK), then acquired by an image analysis system (VersaDoc Imaging System, Bio-Rad Laboratories Inc., Hercules, CA, USA).

Capillary isoelectric-focusing immunoassay

Isoelectric point (pI) analysis of hCG and hMG preparations was performed by capillary isoelectric-focusing immunoassay (cIEF) on a NanoPro™ 100 instrument (ProteinSimple, San Jose, CA, USA), according to the manufacturer's instructions. Briefly, 0.04 µg of hCG/hMG preparations were resuspended in Bicine/CHAPS Lysis and Sample Diluent (20 mM Bicine pH 7.6, 0.6% CHAPS) supplemented with DMSO inhibitor (#040-510, ProteinSimple). Diluted samples were mixed with Premix G2 pH 3-10 (#040-968; ProteinSimple), pI standard Ladder 1 (pI's 4.0, 4.9, 6.0, 6.4 and 7.3; #040-644; ProteinSimple), and pI standard 5.5 (#040-028; ProteinSimple). hCG isoforms characterized by different pIs were revealed by rabbit anti-human polyclonal antibody against hCGβ-subunit (#A0231; Dako, Agilent Technologies). Average exposure time during signal detection was 240 s.

Lectin ELISA assay

The technique was adapted for hCG according to a previous study (Legardinier *et al.*, 2005). A micro-titration plate was coated over-night at 4°C with anti-gonadotropin α subunit monoclonal antibody HT13.3 (Bidart *et al.*, 1988), which recognizes all human α-subunits and glycoprotein hormones, in 0.1 M sodium carbonate/hydrogen carbonate buffer pH = 9.6. The plates was washed by TBS-T buffer (25 mM Tris, 140 mM NaCl, 3 mM KCl, 0.05%, Tween 20; pH = 7.4) and non-specific sites were saturated by 1 h-treatment at room temperature (RT) using TBS-T containing 2% polyvinylpyrrolidone K30 (Fluka, Sigma-Aldrich). Then 1 ng of each hCG preparation was incubated 2 h in 100 µl/well of this saturation buffer, in duplicate. After washing, biotinylated lectins (Vector laboratories Ltd, AbCys Biologie, Paris, France) were placed in the wells for 2 h at RT. The lectins used were: *Sambucus nigra* agglutinin (SNA), *Maackia amurensis* agglutinin (MAA), jacalin, ricin, wheat germ agglutinin (WGA) and succinylated WGA. They were diluted in saturation buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. The plate was washed and peroxidase labelled NeutrAvidin™ (Pierce, Interchim, Montluçon, France) was added in each well (100 µl in TBS-T), for 1 h at RT. After addition of TMB ELISA peroxidase substrate standard solution (UP664781; Interchim, Montluçon, France) for 15 min at

RT, the reaction was stopped with 50 µl/well 2 N H_2SO_4, and absorbance was measured at a 450 nm wave length using a spectrophotometer. Values from blanks were substracted from the samples.

Evaluation of cAMP production and β-arrestin recruitment by BRET

BRET methods for evaluation of cAMP and β-arrestin recruitment were previously validated (Ayoub *et al.*, 2015). Briefly, cAMP production was evaluated in living cells by transiently transfecting HEK293 cells by plasmids carrying the *LHCGR* gene (provided by Professor Alfredo Ulloa-Aguirre, Universidad Nacional Autónoma de México, Mexico City, Mexico) and the BRET-based cAMP sensor CAMYEL (provided by Dr Lily I. Jiang, University of Texas, Dallas, TX, USA). CAMYEL sensor is composed by an inactive cytosolic mutant form of human Epac-1 fused with Renilla luciferase (Rluc) and the green fluorescent protein (GFP) (Jiang *et al.*, 2007; Ayoub *et al.*, 2015). Upon stimulation by hormone, cAMP binding to exchange proteins activated by cAMP (Epac) results in conformational rearrangements of Rluc-Epac-GFP sensor, leading to a dose-dependent decrease of BRET signal. To assess β-arrestin 2 recruitment, HEK293 cells were transiently transfected with plasmid carryng C-terminal Rluc-tagged *LHCGR* gene (provided by Dr Aylin C. Hanyaloglu, Imperial College, London, UK) and N-terminal yPET-tagged β-arrestin 2 (provided by Dr Mark G. Scott, Cochin Institute, Paris, France). Upon hormone stimulation, β-arrestin 2 translocates to LHCGR leading to a BRET signal increase. For both cAMP and β-arrestin 2 assays, HEK293 were incubated for 30 min in a total volume of 40 µl/well PBS and 1 mM Hepes, in the presence or in the absence of increasing hCG/hMG doses (10^{-5}–10^{1} µg/ml range). Upon addition of 10 µl/well of 5 µM Coelenterzine h (Interchim, Montluçon, France), BRET measurements were performed using Mithras LB 943 plate reader (Berthold Technologies GmbH & Co., Wildbad, Germany).

Evaluation of cAMP production by ELISA

hGLC were seeded in 24-well plates (5×10^4 cells/well) and serum-starved 12 h before treatment. Cells were stimulated for 3 h by increasing doses of hCG/hMG preparations (10^{-5}–10^{1} µg/ml range), in the presence of 500 µM 3-isobutyl-1-methylxanthine (IBMX) (#I5879; Sigma-Aldrich), as a phosphodiesterases inhibitor (Lindsey and Channing, 1978). Total cAMP production was evaluated by ELISA (Cyclic AMP Direct EIA kit, Arbor Assays®, Michigan, USA), following supplier's instructions, and absorbance values were measured by Victor3 multilabel plate reader (Perkin Elmer Inc., Waltham, MA, USA). Data were entered into a curve fitting software and represented using a log regression analysis, as previously described (Casarini *et al.*, 2016b).

Evaluation of progesterone synthesis

hGLC were seeded in 24-well plates (5×10^4 cells/well) and serum-starved 12 h before treatment. Cells were stimulated for eight or 24 h by increasing doses of hCG/hMG (10^{-7}–10^{1} µg/ml range). The stimulations were blocked by immediate freezing of the samples, then total progesterone was measured in the supernatants by an immunoassay analyser (ARCHITECT second Generation Progesterone system; Abbot Diagnostics, Chicago, IL, USA).

Statistical analysis

BRET data were represented as 'induced BRET changes' by subtracting the untreated cells 540/480 nm ratio from the values from stimulated cells. Steroid concentrations were represented as ng/ml. All the results were expressed as mean ± SEM and Mann–Whitney's *U*-test or two-way ANOVA and Bonferroni post-test were performed as appropriate. Values were considered statistically significant for $P < 0.05$, but lower P values were also indicated. Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Results

Inference of hCG/hMG preparations weight (in grams) from immunoreactivity

The quantification of hCG, in terms of weight units per volume, is crucial to properly compare their molecular diversity and activity *in vitro*. Indeed, commercial preparations are expressed as IU, as an extrapolation of their biological potency measured *in vivo* (Bangham and Grab, 1964). This parameter depends on molecule half-life *in vivo*, occurring as a result of hCG/hMG molecular heterogeneity and isoforms composition (Furuhashi *et al.*, 1995). Since these molecular features can not be assumed to be homogeneous among the preparations, a comparison of hCG/hMG activities *in vitro* between molecules dosed in IU, determined by *in-vivo* bioassay, is inaccurate. Therefore, the weight of each preparation should be determined by re-calibration against a hCG dosed in weight units (e.g. rhCG Ovitrelle® 250 µg), which was used as a standard to infer the weight (g) from immunoreactivity (IU).

For this purpose, a specific method for hCG quantification was used. Firstly, immunoreactivities of hCG/hMG preparations were determined within the same immunoassay, using a monoclonal antibody against the hCGβ subunit. mIU/ml dosage was extrapolated by data interpolation against the multi-point calibration curve of the hCG standard (Fifth IS; NIBSC 07/364), as expected by the assay and described in the 'Materials and Methods' section. Since the weight of rhCG Ovitrelle® in micrograms is provided by the supplier, this preparation was used to re-calibrate the others, assuming the same immunoreactivity of all the preparations. Specifically, we found that 9544.0 ± 103.3 mIU/ml of rhCG Ovitrelle®, in terms of 'hCG standard Fifth IS; NIBSC 07/364', corresponds to 384.6 ± 4.2 µg/ml (Table I). Therefore, rhCG Ovitrelle® was used to calculate the proportion 1 mIU ~ 0.04 µg, which served to calibrate the weight of the other preparations.

Western blotting and silver staining analysis

Four different hCG and one hMG commercial preparations, quantified by immunoreactivity (Table I), were loaded onto 12% SDS-PAGE under both denaturing (by heating)-reducing and non-denaturing-reducing conditions. hCGβ-related molecules and whole protein content were detected by Western blotting and silver staining (Fig. 1). rFSH Gonal-F® and rLH Luveris® were used as controls and representative pictures from four independent experiments were shown. Under denaturing-reducing conditions, rhCG Ovitrelle® displayed four bands at about 20, 25, 32 and 35 KDa. The 25 and 35 KDa bands are shared with the other preparations (Fig. 1A). Under these conditions, no bands were detected in LH and FSH samples. Under non-denaturing-reducing conditions, up to four hCG protein bands characterized by different intensity and falling at the 25, 32, 37 and 45 KDa molecular weights were detected (Fig. 1B), although the preparation-specific pattern demonstrated by proteins under denaturing-reducing conditions (Fig. 1A) confirmed the diversity of sample composition. The 45 and 32 KDa bands were in all samples. rLH signals at about 35 and 20 KDa demonstrated the hCGβ antibody cross-reactivity to LH heterodimer and its β-subunit, as declared by the supplier. Anti-hCGβ antibody specificity was validated by its pre-absorption with 30 µg

Table I hCG immunoreactivity and quantification of commercial preparations (means ± SEM).

Preparation	mIU-batch loaded	Immunoreactivity (mIU/ml)	Quantification (µg/ml)
Ovitrelle®	5000	9544.0 ± 103.3	384.6 ± 4.2
Gonasi HP®	5000	20 597.5 ± 831.0	830.0 ± 33.5
Pregnyl®	5000	4597.5 ± 197.4	185.3 ± 8.0
C0434	5000	5112.5 ± 144.2	206.0 ± 5.8
Menopur®	5000	757.0 ± 19.0	30.5 ± 0.8
Luveris®	5000	Not detectable	Not available
Gonal-F®	5000	Not detectable	Not available

rhCG Ovitrelle®, revealing the lack of hCG-specific signals, except for the Sigma-Aldrich C0434 50 KDa band (Supplementary Fig. 1).

Silver staining confirmed the patterns observed under both denaturing-reducing and non-denaturing-reducing conditions by Western blotting (Fig. 1C and D). Menopur® and Pregnyl® exhibited a more heterogeneous migration profile, characterized by several protein bands at different molecular weights. Moreover, the presence of FSH in the preparations was evaluated by Western blotting (Fig. 1E and F). As expected, the FSH band was detected only in hMG Menopur® and confirmed by rFSH Gonal-F® (positive control), as well as by immunoassay (Table II). Lower intensities of FSH bands were observed under non-denaturing-reducing conditions, compared to denaturing-reducing conditions, likely as an effect of decreased accessibility of epitopes to antibody binding. Moreover, the absence of LH signals detectable by silver staining, under denaturing-reducing conditions (Fig. 1C), matches the result obtained by Western blotting (Fig. 1A) and it may be plausibly attributable to the loss of epitopes by denaturation.

Analysis of isoelectric points

pI was evaluated by cIEF in gonadotropin preparations, revealing specific spectrum of pI values (pI = 4.0–9.0 range) with most of the isoforms falling in the acidic range (pI < 7.0) (Fig. 2). Raw pI and percentage values of each peak, as a proportion of the whole preparation profile, are presented in Table III. All the preparations resulted in a similar pattern of peaks falling within the acidic pH range, with six peaks representing pI < 5.0. Therefore, the Menopur® isoelectric pattern is consistent with the presence of hCG in the preparation. Interestingly, Gonasi® and C0434 displayed further peaks at a higher pH range than other preparations (pI = 6.0–9.0), reasonably corresponding to less glycosylated hCG isoforms. Finally, the heterogeneity of peak widths among the preparations revealed that gonadotropin isoforms are quantitatively, differently represented.

Reactivity of gonadotropin preparations to lectin binding

The carbohydrate structure of hCG/hMG preparations was investigated by lectin assay, using a panel of six molecules featured by specific affinity to different glycans (Table IV). Lectin binding to gonadotropin carbohydrate side chains was evaluated by ELISA. We also tested natural pituitary human LH and FSH as these two gonadotropins are present in Menopur® and recognized by the coated human anti-α subunit antibody HT13.3. Each preparation displayed different affinity to lectins, revealing distinct glycosylation profiles and binding patterns (Fig. 3). All tested hCGs reacted with MAA, specific for Neu5Acα2-3Gal glycan structure (Wang and Cummings, 1988). Interestingly, in our experimental conditions, only Menopur® reacted with SNA, which is specific for Neu5Acα2-6Gal (Shibuya *et al.*, 1987). This can be attributed to the presence of human FSH and LH which interact with SNA (Supplementary Fig. 2). Jacalin reacted with all hCG preparations, demonstrating the presence of *O*-glycan chains (Roque-Barreira and Campos-Neto, 1985; Kabir, 1998; Tachibana *et al.*, 2006; Bai *et al.*, 2015). Moreover, the jacalin signals detectable in Gonasi®, Pregnyl®, C0434 and Ovitrelle® preparations was significantly higher than in Menopur ($P < 0.001$; two-way ANOVA and Bonferroni post-test; $n = 7$). No jacalin binding was shown with natural human LH and FSH which are devoid of *O*-glycan chain, showing that only the hCG fraction of Menopur is detected (Supplementary Fig. 2). WGA reacted with all five preparations tested, revealing lower signal intensity in Menopur® vs C0434 and Ovitrelle® (two-way ANOVA and Bonferroni post-test; $P < 0.05$; $n = 7$). Binding capability dramatically decreased using the WGA succinylated analog, suggesting the presence of sialic acid (Bhavanandan and Katlic, 1979; Monsigny *et al.*, 1979, 1980). Ricin binding was observed in all the preparations, especially in Menopur®, where the signal achieved higher levels than in Pregnyl® and C0434 (two-way ANOVA and Bonferroni post-test; $P < 0.05$; $n = 7$), indicating the presence of non-sialylated terminal Galβ1-4GlcNAc structures on glycan chains (Green *et al.*, 1987). The garaph in Fig. 3 is alternatively displayed according to the lectins (Supplementary Fig. 3).

Analysis of cAMP production

Preparation-specific intracellular cAMP production was assessed in living HEK293 cells transiently co-expressing with LHCGR and the BRET-based cAMP sensor by 30-min dose–response experiments (Fig. 4). Gonasi®, Menopur® and C0434 treatment resulted in significantly higher half-maximal effective concentrations (EC_{50}) compared to rhCG Ovitrelle® (Mann–Whitney's *U*-test; $P < 0.05$; $n = 4$). Moreover, only C0434 exhibited lower efficacy than Menopur® (Mann–Whitney's *U*-test; $P < 0.05$; $n = 4$), measured as maximal levels (E_{max}) of 'induced BRET changes'. Control experiments evaluating FSH activity of Menopur® demonstrated that this preparation induces cAMP synthesis in FSHR-transfected HEK293 cells, as compared to Gonal-F® (Supplementary Fig. 4). Ovitrelle® served as a negative control and failed to induce cAMP production in this setting.

Total cAMP was also measured in hGLC, naturally expressing LHCGR (and FSHR). Dose–response experiments were performed and cAMP production was evaluated by ELISA after 3 h of treatment (Fig. 4B). Pregnyl® and Menopur® displayed lower potency than rhCG Ovitrelle®, in terms of cAMP accumulation, as shown by the different EC_{50} values (Mann–Whitney's *U*-test; $P < 0.05$; $n = 5$). The same graph expressing gonadotropins in IU/ml (Supplementary Fig. 5) does not provide substantial different dose–response curves.

β-arrestin 2 recruitment

β-arrestin 2 recruitment induced at the LHCGR upon treatment with the different preparations, was evaluated in HEK293 cells co-expressing with LHCGR tagged to *Renilla* luciferase (LHCGR-Rluc) and

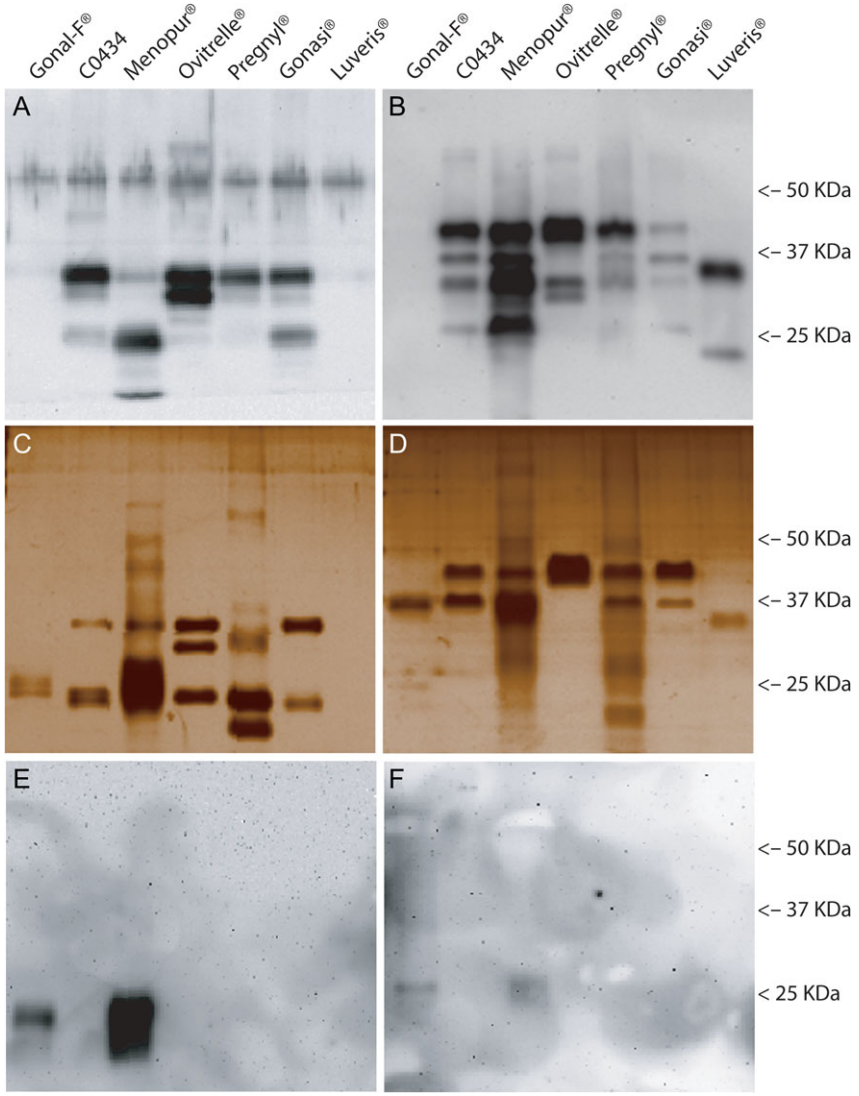

Figure 1 Western blotting and silver staining analysis of hCG/hMG preparations under native and denaturing reducing conditions. hCG and hFSH isoforms were detected by rabbit anti-hCGβ polyclonal antibody, which retains 4% hLH cross-reactivity, and rabbit anti-hFSH polyclonal antibody. Preparations of recombinant hFSH and hLH were used as controls. (**A**) Evaluation of preparations under denaturing-reducing conditions, by Western blotting, using anti-hCGβ antibody. These concentrations of preparations were loaded: 0.15 μg Gonal-F®; 0.50 μg C0434; 0.15 μg Menopur®, Ovitrelle®, Pregnyl® and Gonasi®; 0.15 μg Luveris®. (**B**) Analysis of preparations under native conditions by Western blotting. Anti-hCGβ antibody was used. Concentrations: 0.15 μg Gonal-F®; 0.15 μg C0434; 0.15 μg Menopur®; 0.005 μg Ovitrelle®, Pregnyl® and Gonasi®; 0.15 μg Luveris®). (**C**) Silver staining of preparations under denaturing-reducing conditions. Concentrations: 3.00 μg Gonal-F®; 3.00 μg C0434; 0.70 μg Menopur®, Ovitrelle®, Pregnyl® and Gonasi®; 3.00 μg Luveris®. (**D**) Preparations under native conditions evaluated by silver staining. Concentrations: 3.00 μg Gonal-F®; 3.00 μg C0434; 0.70 μg Menopur®, Ovitrelle®, Pregnyl® and Gonasi®; 3.00 μg Luveris®. (**E**) Western blot of preparations under denaturing-reducing conditions, using anti-hFSH antibody. Concentrations: 3.00 μg Gonal-F®; 3.00 μg C0434; 0.70 μg Menopur®, Ovitrelle®, Pregnyl® and Gonasi®; 3.00 μg Luveris®. (**F**) Western blotting analysis of preparations under native conditions. Anti-hFSH antibody was used. Concentrations: 3.00 μg Gonal-F®; 3.00 μg C0434; 0.70 μg Menopur®, Ovitrelle®, Pregnyl® and Gonasi®; 3.00 μg Luveris®. Pictures are representative of four independent experiments.

β-arrestin 2 tagged to a variant of the yellow fluorescent protein (β-arrestin 2-yPET). Cells were treated by increasing doses of hCG/hMG preparations and β-arrestin 2 recruitment was evaluated by BRET after 30-min treatment (Fig. 5). Gonasi® and C0434 treatment resulted in less potent β-arrestin 2 recruitment compared to Ovitrelle®, as reflected by higher EC_{50} values demonstrated (Mann–Whitney's *U*-test; $P < 0.05$; $n = 4$). The partial agonism of C0434 in recruiting β-arrestin 2 was observed as it led to a significantly lower E_{max} than Ovitrelle® (Mann–Whitney's *U*-test; $P < 0.05$; $n = 4$). No significantly different potency or efficacy was found between Menopur® and Ovitrelle®, in terms of β-arrestin 2 recruitment, likely due to the low number of dose-points achievable.

Table II LH/FSH immunoreactivity and quantification of commercial preparations (means ± SEM).

Preparation	LH Immunoreactivity (mIU/ml)	FSH Immunoreactivity (mIU/ml)
Ovitrelle®	Not detectable	Not detectable
Gonasi HP®	Not detectable	Not detectable
Pregnyl®	Not detectable	Not detectable
C0434	Not detectable	Not detectable
Menopur®	30.8 ± 1.0	2555.7 ± 14.0
Luveris®	1553.7 ± 64.0	Not detectable
Gonal-F®	Not detectable	1166.7 ± 3.9

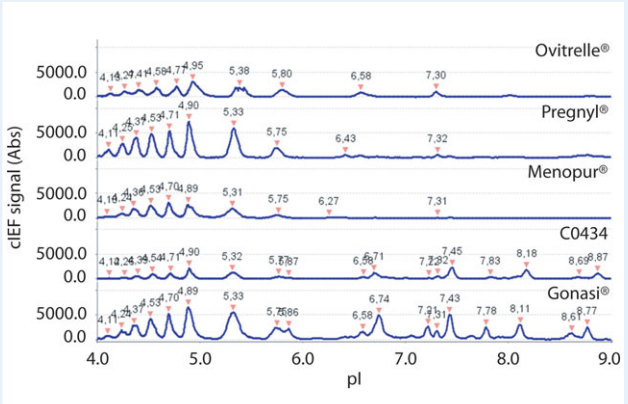

Figure 2 hCG isoforms separation by capillary isoelectric-focusing immunoassay. Isoelectric points (pI) of 0.04 µg hCG/hMG preparations were analyzed revealing different rates of acidic residues. hCG isoforms were detected by rabbit anti-hCGβ polyclonal antibody. Peaks represent increasing acidic hCG isoforms, according to the direction right-to-left on *x*-axis.

Evaluation of progesterone production

In hGLC, progesterone synthesis is a cAMP-dependent, physiological event mediated by gonadotropins. Progesterone production was evaluated in 8-h and 24 h-stimulated cells by the drugs, and the steroid was measured using immunoassay (Fig. 6). Reflecting cAMP production, hCG/hMG preparations differently triggered the early (8-h) progesterone accumulation (Fig. 6A), revealing different steroidogenic potential retained by the preparations. However, after 24 h of continuous stimulation by gonadotropins, progesterone production achieved a plateau, as previously observed (Nordhoff *et al.*, 2011; Casarini *et al.*, 2014, 2016b). Indeed, no significant differences in the 24-h progesterone production were observed among the preparations, demonstrated by the dose–response curves and, accordingly, by similar EC_{50} and E_{max} values (Mann–Whitney's *U*-test; $P \geq 0.05$; $n = 3$).

Discussion

In this study, we compared the biochemical compositions and *in-vitro* activities of four hCG and one hMG preparations. Except for C0434, which was developed for research use only, these hormones are used together with FSH in ART to promote folliculogenesis and trigger ovulation (Casarini *et al.*, 2016a). For this purpose, several hCG preparations are commercially available, differing in source and purification procedures, resulting in multiple isoform combinations. While Ovitrelle® is a human recombinant preparation obtained from transfected CHO cells, Gonasi® and Pregnyl® are products purified from urine of pregnant women. C0434 is a hCG developed for research, instead of clinical purpose, and purified from pregnancy urine as well. As claimed by the Supplier in the drug's leaflet, Menopur® is a purified mixture of human menopausal gonadotropins, therefore containing pituitary hCG, differently from the other preparations which feature hCG of trophoblast origin. This is in contrast with a previous report assuming that 'relatively high amount of hCG can only be explained by assuming the addition of HCG from external sources' in Menopur® (van de Weijer *et al.*, 2003), while another study suggested that hCG from pregnant women is added only when it is necessary to achieve the 1:1 FSH to LH activity ratio (Di Stefano *et al.*, 2016). In any case, the inclusion of Menopur® may provide information on plausible, different compositions and cell responses *in vitro* between recombinant/extractive gonadotropins of pituitary or trophoblast origin, especially in HEK293 cells, not expressing FSHR. Preparations are expressed in IU obtained by *in-vivo* bioassays (Storring *et al.*, 1981, 1982), where steroid-dependent endpoints are evaluated (e.g. organ weight) and a different gonadotropin-specific signaling due to rodent receptors may occur (Riccetti *et al.*, 2017a). Therefore, to analyze and compare their induced-signaling *in vitro*, we first determined the hCG molar quantities of each preparation by immunoassay against a standard and assuming similar immunoreactivity of different isoproteins (Berger and Lapthorn, 2016). This approach revealed wide heterogeneity in hCG quantification among the preparations. In particular, FSH and LH immunoreactivity demonstrated in Menopur® (van de Weijer *et al.*, 2003) supports that these gonadotropins contribute to the drug activity *in vivo*. In addition, hCGβ subunits were evaluated by Western blotting under both native and denaturing-reducing conditions, revealing specific profiles consisting of a number of hCG bands, ranging between the molecular weights of 20–50 KDa. Under native conditions, a 45 KDa hCG isoform was abundant among all the preparations, as a likely hyperglycosylated heterodimer. The presence of 45 KDa hCG proteins was confirmed by silver staining. Additional bands were also detected in Pregnyl® and Menopur® preparations potentially corresponding to distinct isoforms or degradation products. These results support previous studies reporting at least six hCG variants in addition to the 37 KDa 'classical' choriogonadotropin (Kovalevskaya *et al.*, 1999; Butler *et al.*, 2001; Cole, 2012b).

Heterogeneous patterns of pI values were detected by cIEF. Given that sugar structures contribute to increased acidity of hCG, the analysis of isoelectric point suggests the presence of different glycosylation isoforms among drugs. Each hormone exhibited six peaks within the acidic pH range (pI < 5), while the extractive preparations Gonasi® and C0434 were characterized by additional peaks with more alkaline profiles (pI > 6) reasonably corresponding to poorly glycosylated hCG isoforms. Isoelectric pattern of gonadotropin preparations depends on the purification procedures (Storring *et al.*, 1982), physiological status of the donor subject (Robertson *et al.*, 1977; Van Damme *et al.*, 1977; Wide, 1985), as well as generation of degradation artefacts (Zaidi *et al.*, 1981), leading to preparations with multiple hCG variants whose

Table III Isoelectric profile peaks of commercial preparations (means±SD).

Ovitrelle®		Pregnyl®		Menopur®		Gonasi®		C0434	
pI ± ΔpI	% pI ± SD	pI ± ΔpI	% pI ± SD	pI ± ΔpI	% pI ± SD	pI ± ΔpI	% pI ± SD	pI ± ΔpI	% pI ± SD
4.13 ± 0.01	4.65 ± 2.20	4.11 ± 0.01	5.11 ± 0.20	4.10 ± 0.00	2.53 ± 0.87	4.12 ± 0.01	0.71 ± 0.31	4.10 ± 0.01	1.85 ± 0.15
4.27 ± 0.01	8.20 ± 0.75	4.25 ± 0.01	9.62 ± 0.44	4.24 ± 0.01	6.21 ± 0.20	4.26 ± 0.01	1.73 ± 0.40	4.26 ± 0.01	4.28 ± 0.13
4.41 ± 0.01	11.93 ± 1.50	4.37 ± 0.01	14.15 ± 0.60	4.36 ± 0.01	14.03 ± 0.04	4.39 ± 0.01	4.58 ± 0.55	4.39 ± 0.01	8.24 ± 0.41
4.58 ± 0.01	11.58 ± 0.99	4.53 ± 0.01	15.95 ± 0.34	4.53 ± 0.01	18.83 ± 0.24	4.54 ± 0.01	7.36 ± 0.72	4.52 ± 0.01	11.13 ± 0.68
4.77 ± 0.01	11.52 ± 1.65	4.71 ± 0.01	14.06 ± 0.33	4.70 ± 0.01	17.48 ± 0.41	4.71 ± 0.01	8.24 ± 0.61	4.70 ± 0.01	11.35 ± 0.96
4.95 ± 0.00	17.20 ± 4.26	4.90 ± 0.01	18.51 ± 0.21	4.89 ± 0.01	20.35 ± 0.98	4.90 ± 0.01	15.30 ± 3.19	4.89 ± 0.01	15.62 ± 1.68
5.38 ± 0.01	18.06 ± 3.00	5.33 ± 0.02	16.63 ± 1.01	5.31 ± 0.00	15.44 ± 0.37	5.32 ± 0.00	14.24 ± 0.50	5.32 ± 0.00	15.97 ± 0.51
5.80 ± 0.02	6.50 ± 0.31	5.75 ± 0.02	4.23 ± 0.55	5.75 ± 0.01	3.85 ± 0.17	5.77 ± 0.01	3.61 ± 0.39	5.74 ± 0.01	3.85 ± 0.67
6.58 ± 0.00	4.45 ± 0.75	6.43 ± 0.02	0.99 ± 0.09	6.27 ± 0.04	1.04 ± 0.28	5.87 ± 0.00	0.96 ± 0.07	5.86 ± 0.00	2.20 ± 0.28
7.30 ± 0.01	3.54 ± 0.79	7.32 ± 0.02	0.74 ± 0.31	7.31 ± 0.02	0.23 ± 0.04	6.58 ± 0.01	1.30 ± 0.14	6.58 ± 0.01	2.25 ± 0.36
						6.71 ± 0.03	9.80 ± 3.33	6.74 ± 0.03	5.77 ± 1.19
						7.22 ± 0.01	0.38 ± 0.11	7.21 ± 0.01	2.45 ± 0.16
						7.32 ± 0.01	2.33 ± 0.46	7.31 ± 0.01	1.25 ± 0.05
						7.45 ± 0.02	9.89 ± 0.75	7.43 ± 0.01	4.99 ± 0.33
						7.83 ± 0.05	1.85 ± 0.16	7.78 ± 0.03	2.00 ± 0.38
						8.18 ± 0.06	9.25 ± 0.70	8.11 ± 0.03	3.16 ± 0.61
						8.69 ± 0.08	2.64 ± 0.45	8.61 ± 0.04	1.73 ± 0.53
						8.87 ± 0.11	6.04 ± 0.76	8.77 ± 0.04	1.95 ± 0.46

Table IV Lectins specific affinity to different glycans.

Lectins	Name	Organism	Binding site
MAA	*Maackia amurensis* agglutinin	*Maackia amurensis*	Neu5Acα2-3Gal (N-or 0-glycan)
SNA	*Sambucus nigra* agglutinin	*Elderberry bark*	Neu5Acα2-6Gal (NAc) (N-or 0-glycan)
WGA	Wheat germ agglutinin	*Tritricum vulgaris*	GlcNAc, Neu5Ac (N- or 0-glycan)
Succinylated WGA	Succinylated wheat germ agglutinin	*Tritricum vulgaris*	GlcNAc (N- or 0-glycan)
Jacalin	Jacalin	*Artocarpus integrifolia*	(Neu5Ac)Galb1-3GalNAc, GalNAc (0-glycan)
Ricin (RCA_{120})	*Ricinus communis* agglutinin 120	*Ricinus communis*	Galβ1-4GlcNAc (N- or 0-glycan)

biological activity remains a debated matter (Arey and López, 2011). In particular, a spectrum of differently glycosylated hCGs were found over the first trimester of pregnancy, when these molecules plausibly mediate trophoblast invasion and cell proliferation (Evans *et al.*, 2015). Previous studies demonstrated a wide range of hCG isoforms in plasma, urine or in media of cultured cells (Berger *et al.*, 1993; Díaz-Cueto *et al.*, 1994), when attempting to isolate the molecules corresponding to each peak and to investigate their effects *in vitro* (Ulloa-Aguirre *et al.*, 1990). Further analysis of hCG drugs by combining mass-spectrometry, tandem mass tag labeling and a multivariate analysis, revealed different O- and N-linked glycans between urinary and recombinant gonadotropins, moreover, providing a method suitable for glycoprotein characterization (Zhu *et al.*, 2017). More basic isoforms mediate higher cAMP and/or steroids production increases than more acidic variants, as shown by our finding in hGLC using Gonasi® (Figs 4 and 6), likely due to higher receptor affinity (Ulloa-Aguirre *et al.*, 1990; Sairam and Jiang, 1992; Bousfield *et al.*, 2004). However, these molecules should be analyzed separately in *in-vitro* models to fully understand their activity.

The glycosylation type was evaluated in gonadotropin preparations by reactivity to six lectins. Human α subunit bears two *N*-glycan chains while hCGβ has two *N*-glycan and four *O*-glycan chains bound to protein backbone (Kobata, 1988). However, variation in glycan structures exist, depending on the hormone source (Bousfield *et al.*, 2014b). We show sialylation in α2–3 position on terminal Galactose of the tested hCG extracted from urine. Sialyl α2–6 osberved in Menopur® may be attributed to FSH present in this preparation. Glycosylation of recombinant gonadotropins depends on the cellular type used to produce them: CHO-K1 cells do not sialylate at position 2–6 of terminal galactose because of a lack of galactoside α-2,6-sialyltransferase (ST6Gal) gene expression (Xu *et al.*, 2011). Heterogeneity of peaks obtained in IEF might be explained by different sialylation rate of the preparations, which are associated with the presence of desialylated glycan chains. Jacalin is also an useful tool to evaluate *O*-glycosylation of hCG

(Bai *et al.*, 2015). In our experimental conditions, it detects hCG in Menopur® while human FSH and LH do not bind jacalin. Indeed, the preparations displayed different pattern of affinity to lectins. ing data have been reported about the role of sugar moieties on receptor binding and hormone activity *in vitro* (Browne *et al.*, 1990; Bishop *et al.*, 1994; Bousfield *et al.*, 2004; Wehbi *et al.*, 2010), suggesting that they may impact half-life and immunoreactivity *in vivo* (Fares, 2006). Moreover, in Menopur®, the presence of FSH and LH molecules may result in modulation of hCG-specific cell responses *in vitro*, as previously suggested (Zhang *et al.*, 2009; Casarini *et al.*, 2016b). The relevance of gonadotropin glycosylation variants on receptor binding and cell signaling activation *in vitro*, as well as their physiological role *in vivo*, is a controversial matter currently debated. Several studies have attempted to characterize the activity of different hCG glycoforms secreted during pregnancy (Guibourdenche *et al.*, 2010; Berndt *et al.*, 2013; Fournier *et al.*, 2015), as well as that of hypo- and fully glycosylated human FSH, both *in vitro* and in FSHβ-knock out mice *in vivo* (Wehbi *et al.*, 2010; Bousfield *et al.*, 2014a; Wang *et al.*, 2016).

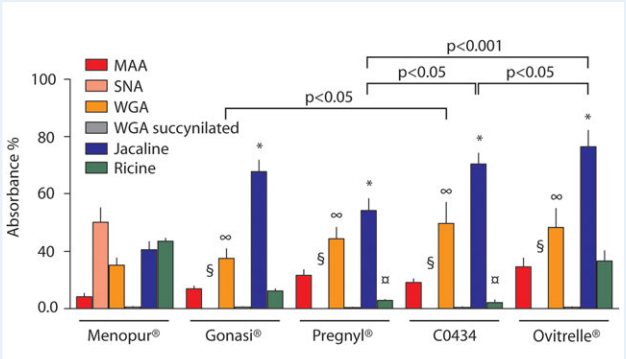

Figure 3 Comparison of hCG/hMG preparations affinity to lectins. hCG/hMG lectin binding pattern was evaluated by ELISA. Data are represented as means ± SEM. Differences were considered significant for $P < 0.05$ (two-way ANOVA and Bonferroni post-tests; $n = 7$). * = different vs Menopur®-Jacaline signal ($P < 0.001$); § = different vs Menopur®-SNA signal ($P < 0.001$); ¤ = significantly different vs Menopur-ricine signal ($P < 0.01$); ∞ = different vs Menopur®-WGA signal ($P < 0.05$). Brackets show specific differences between preparations.

We proceeded by analyzing hCG/hMG preparations-induced cell signaling in terms of cAMP production, β-arrestin 2 recruitment and the downstream steroidogenesis in either HEK293 cells transiently expressing LHCGR and hGLC *in vitro*. All the preparations induced cAMP accumulation and β-arrestin 2 recruitment in the pM and nM range, respectively, confirming the hCG dose shift between the two intracellular endpoints, as previously demonstrated (Ayoub *et al.*, 2015; Riccetti *et al.*, 2017b). rhCG Ovitrelle® exhibited higher potency but similar efficacy in inducing cAMP accumulation than some urinary preparations, in both cell models (Figs 4 and 6). Interestingly, gonadotropin-specific cAMP production may be dependent on the source cell, i.e. CHO cells for Ovitrelle® and trophoblast or pituitary cells for urinary preparations, leading to specific patterns of post-translational modification of the molecules. Ovitrelle® and Pregnyl® were more potent than Gonasi® and C0434 in triggering β-arrestin 2 recruitment (Fig. 5). Interestingly, C0434 displayed partial agonism in both cAMP production and β-arrestin 2 recruitment, as shown by its lower E_{max} than that of the other drugs. This matches with the heterogenous signals obtaind by Western blotting, silver staining and cIEF for C0434, suggesting the presence of contaminants and/or partially degraded forms in the preparation. In addition, differences in hCG isoform composition might be responsible of these different pharmacological activities. Previous comparisons between LH and hCG in

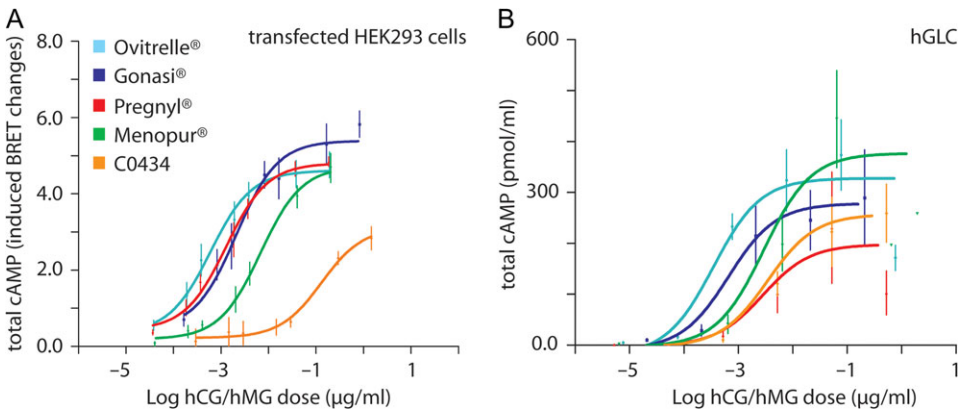

Figure 4 cAMP response induced by hCG/hMG preparations in transfected HEK293 and hGLC. Cells were treated by increasing concentrations of hCG/hMG before cAMP measurement. (**A**) Intracellular cAMP increase in HEK293 cells transiently co-transfected with LHCGR and CAMYEL sensor. cAMP was measured by BRET after 30 min. Significantly different EC50s are between Ovitrelle® (0.00078 ± 0.00018 µg/ml) vs Gonasi® (0.0034 ± 0.0012 µg/ml), $P = 0.0294$; Ovitrelle® vs Menopur® (0.0094 ± 0.0010 µg/ml), $P = 0.0294$; Ovitrelle® vs C0434 (0.1400 ± 0.0300 µg/ml), $P = 0.0286$; means ± SEM, Mann–Whitney's *U*-test, $n = 4$. Significantly different E_{max} are between Ovitrelle® (0.46 ± 0.040) vs C0434 (0.3100 ± 0.0360 induced BRET changes), $P = 0.0366$; means ± SEM, Mann–Whitney's *U*-test, $n = 4$. (**B**) Total cAMP increase, measured by ELISA, in 3-h stimulated hGLC. Data were interpolated by nonlinear regression. Significantly different EC50s are between Ovitrelle® (0.006 ± 0.0024 µg/ml) vs Pregnyl® (1.0000 ± 0.9500 µg/ml), $P = 0.0317$; Ovitrelle® vs Menopur® (0.05600 ± 0.01200), $P = 0.0159$; means ± SEM, Mann–Whitney's *U*-test, $n = 5$.

human and goat primary granulosa cells revealed that the two molecules are not equivalent *in vitro*, resulting in higher hCG- than LH-mediated steroidogenic and pro-apoptotic activity, while LH preferentially activates proliferative and anti-apoptotic signals (Casarini *et al.*, 2012, 2016b; Gupta *et al.*, 2012). These data demonstrated that ligand-dependent signaling may be exerted by the same receptor. hCG-specific activity *in vitro* was amplified in the presence of 10 nM FSH, suggesting FSHR/LHCGR heterodimerization and cross-talk between intracellular signaling pathways (Casarini *et al.*, 2016a). However, we found that Menopur® did not result in a more potent or efficient cell signaling activation than the other hCGs. This could potentially reflect balanced FSH and LH co-activity, or sub-optimal FSH dosage for *in-vitro* evaluations.

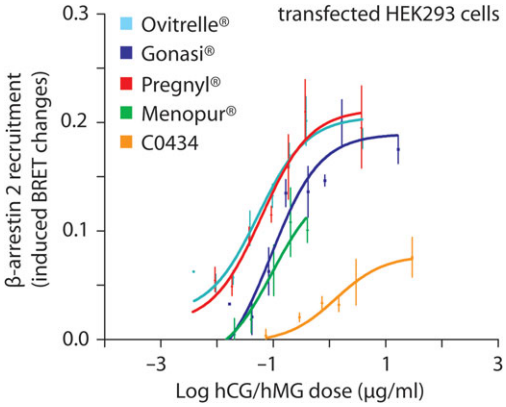

Figure 5 β-arrestin 2 recruitment in transfected HEK293 cells. Cells were transiently co-transfected with LHCGR-Rluc8 and β arrestin-2-YPET sensor, then they were treated by increasing doses of hCG/hMG preparations. β arrestin-2 recruitment was measured by BRET upon 30-min stimulation. Data were interpolated by non-linear regression. Significantly different EC50s are between Ovitrelle® (0.0500 ± 0.0085 µg/ml) vs Gonasi® (0.19 ± 0.0060 µg/ml), $P = 0.0286$; Ovitrelle® vs C0434 (1.1100 ± 0.4100 µg/ml), $P = 0.0294$; (Mann–Whitney's *U*-test). Significantly different E_{max} are between Ovitrelle® (0.2000 ± 0.01000 induced RET changes) vs Gonasi® (0.0800 ± 0.0020 induced BRET changes), $P = 0.0294$; means ± SEM, Mann–Whitney's *U*-test, $n = 4$.

Interestingly, looking at the hCG-induced progesterone responses after 8 h treatment, the differences measured between the preparations were in line with cAMP production, highlighting the contribution of glycoforms composition in modulating the early steroids production downstream cAMP/PKA pathway activation (Fig. 6A). This is consistent with previous studies demonstrating matching between intracellular signaling and steroid synthesis *in vitro* (Casarini *et al.*, 2014, 2016b; Ayoub *et al.*, 2016; Riccetti *et al.*, 2017a). However, hCG/hMG preparations treatment resulted in similar progesterone production after 24 h treatment (Fig. 6B). As previously observed (Casarini *et al.*, 2016b), the long-term-cumulative effect on steroidogenesis upon gonadotropins stimulation could mask the differences observed in upstream signaling induced by the hCG/hMG preparations, resulting in a similar steroidogenic potential observed *in vitro* and *in vivo*. Indeed, the calibration of commercial gonadotropins relies on their *in-vivo* activity (Pharmacopea) and can be reached with different mixtures of isoforms presenting heterogeneous half-lives and receptor binding activities. Here, we demonstrate that commercial hCG preparations, while presenting different assortments of isoforms, ultimately exhibit similar steroidogenic activity *in vitro*, despite triggering different pharmacological activities on upstream signaling events.

Taken together, the converging effects on progesterone synthesis evaluated *in vitro* demonstrate that methods for therapeutic hCG/hMG development and purification result in compounds reliably usable for clinical purpose in human. This is confirmed by studies comparing the impact of these preparations on clinical outcomes, such as live birth rate, pregnancy rate or cases of ovarian hypertimulation syndrome, which are not affected by the source of the hCG/hMG preparation used for ART (Youssef *et al.*, 2016). Interestingly, a specific, gonadotropin-dependent impact on ART outcomes was found by a

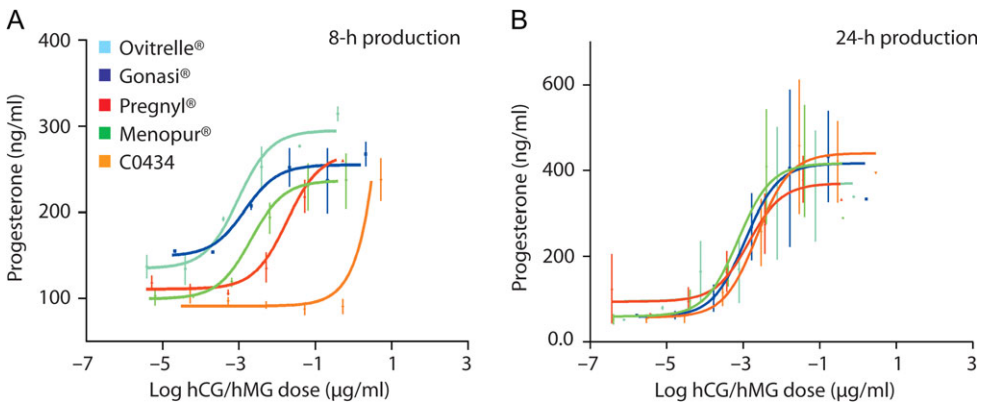

Figure 6 Progesterone production in hGLC treated by hCG/hMG preparations. cAMP accumulation was measured by immunoassay. (**A**) 8-h progesterone production (means ± SEM; $n = 2$). (**B**) 24-h progesterone production ($n = 4$). Data were interpolated by nonlinear regression. No statistically different EC_{50} and E_{max} were found among 24-h results (means ± SEM, $P \geq 0.05$, Mann–Whitney's *U*-test).

recent meta-analysis (Santi *et al.*, 2017). This study evaluating data from a relatively high number of articles ($n = 70$), demonstrated that the highest oocyte number may be achieved using FSH alone. The addition of hMG should improve the recovery of mature oocytes, embryos and implantation rate, while the addition of LH is linked to a higher pregnancy rate. Therfore, hCG/hMG preparations display different intracellular signaling, however without impacting on ART outcomes, while different gonadotropin molecules may reflect specific clinical actions (Santi *et al.*, 2017) according to *in-vitro* data (Casarini *et al.*, 2012, 2016b, 2017; Riccetti *et al.*, 2017a,b). As a future perspective, results from cell signaling analysis may provide information useful for personalized treatment of patients. It is known that single-nucleotide polymorphisms and mutations falling within the gonadotropin receptor genes may modulate the cell response to the hormones, e.g. cAMP production and ovarian stimulation (Tranchant *et al.*, 2011; Casarini *et al.*, 2014). Similarly, pathological conditions, such as polycystic ovary syndrome, may benefit from treatment aimed to specific intracellular targets (Rice *et al.*, 2013), suggesting that gonadotropins mediating specific signal transduction may be suitable for this purpose.

Conclusions

This study demonstrates heterogenous composition of recombinant and urine-derived hCG and hMG preparations, most of which are currently used in ART. The drug mediated different cAMP production and β-arrestin 2 recruitment but did not result in differential progesterone production after 24-h treatment in human primary granulosa cells *in vitro*. However, differences in activating signal transduction pathways and in progesterone production after 8-h treatment suggest that further investigations focused on the kinetics of steroid synthesis mediated by purified isoforms could clarify the contribution of glycosylation on the action of gonadotropins *in vitro*.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

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Authors' roles

L.R. wrote the article, performed experiments, and participated in the study design and data interpretation. D.K. and M.A.A. edited the article, performed and conceived some experiments and contributed to data interpretation. T.B., E.P., S.T., M.V., T.T., A.N., F.C. and G.B.L.S. gave fundamental contributions to editing the article editing or performing experiments. E.R., M.S. and L.C. carried out the study design, article editing, experimental management and data interpretation.

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Conflict of interest

The auhors have no conflict of interest.

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