

# Gonadotropin therapy in assisted reproduction: an evolutionary perspective from biologics to biotech

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Gonadotropin therapy plays an integral role in ovarian stimulation for infertility treatments. Efforts have been made over the last century to improve gonadotropin preparations. Undoubtedly, current gonadotropins have better quality and safety profiles as well as clinical efficacy than earlier ones. A major achievement has been introducing recombinant technology in the manufacturing processes for follicle-stimulating hormone, luteinizing hormone, and human chorionic gonadotropin. Recombinant gonadotropins are purer than urine-derived gonadotropins, and incorporating vial filling by mass virtually eliminated batch-to-batch variations and enabled accurate dosing. Recombinant and fill-by-mass technologies have been the driving forces for launching of prefilled pen devices for more patient-friendly ovarian stimulation. The most recent developments include the fixed combination of follitropin alfa + lutropin alfa, long-acting FSH gonadotropin, and a new family of prefilled pen injector devices for administration of recombinant gonadotropins. The next step would be the production of orally bioactive molecules with selective follicle-stimulating hormone and luteinizing hormone activity.

**KEYWORDS:** Gonadotropins; Ovulation Induction; Assisted Reproductive Techniques; Systematic Review.

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## ■ INTRODUCTION

Gonadotropin therapy plays an integral role in ovarian stimulation for infertility treatments. It was introduced almost one century ago, and the last 25 years have yielded major advancements.

Treating anovulatory women through exogenous gonadotropin administration began in the 1960s. It then expanded to ovulatory women undergoing treatment with assisted reproduction technology (ART) in the 1980s (1-3). In fact, the introduction of controlled ovarian stimulation (COS) for multiple follicular development significantly increased pregnancy rates in *in vitro* fertilization (IVF) (4). Such stimulation protocols have been developed and refined to maximize the beneficial effects of treatment while minimizing complications and risks (5).

In this review, we first describe the gonadotropin glycoprotein structure and actions. We then outline the landmark research that generated the currently used gonadotropins. Next, we critically discuss the quality,

safety, and clinical efficacy of commercially available gonadotropins, and last, we present an overview of the novel pharmaceutical preparations under investigation.

## ■ GONADOTROPIN STRUCTURE AND FUNCTION

The three gonadotropins, follicle-stimulating hormone (FSH), luteinizing hormone (LH) and human chorionic gonadotropin (hCG), are glycoproteins composed of two non-covalently linked protein subunits, the alpha and beta subunits (6). The alpha subunit contains 92 amino acids (AA) and is identical in FSH, LH, and hCG. In contrast, the beta subunits are distinct and confer unique receptor specificity as well as differential biological properties (7). Its biological activity is provided by the attachment of carbohydrate moieties, forming heterodimers (3). The extent and pattern of glycosylation conveys the spectrum of different charges, bioactivities and half-lives for each glycoprotein (8). Glycoproteins have two basic types of glycosylation patterns: O-linked glycosylation, which is characterized by a carbohydrate N-acetylgalactosamine (GalNAc) attached to the hydroxyl group of an amino acid, serine or threonine, and N-linked glycosylation, which is characterized by an N-acetyl glucosamine (GlcNAc) attached to the amide group of asparagine (Asn) (9). The oligosaccharides often terminate with sialic acid and/or sulfonated  $\beta$ 1-4-linked GalNAc ( $\text{SO}_3\text{-4GalNAc}$ ) (10,11). Molecules with a large number of sulfonated GalNAcs disappear faster from the circulation than less-sulfonated isoforms due to their affinity for liver  $\text{SO}_3\text{-4GalNAc}$

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receptors (10,12). On the other hand, more sialic acids enhance the half-life (10,13).

### Follicle-stimulating hormone

The FSH beta subunit is composed of 111 AAs with four N-linked glycosylation sites, two on the alpha subunit (Asn52 and Asn78) and two on the beta subunit (Asn7 and Asn24) (9,14). Thus, each subunit is attached to two carbohydrate moieties with variable compositions that, in turn, create different isoforms with different plasma half-lives (ranging from 3 to 4 hours) and bioactivities (Figure 1) (3,9). Sialic acid residues are much more common in FSH than sulfonated residues (13). Increased sialylation enhances FSH metabolic stability by decreasing both glomerular filtration and clearance by liver sialoglycoprotein receptors, which is the major site for gonadotropin clearance (15,16).

FSH stimulates the recruitment and growth of early antral follicles (2-5 mm in diameter) by binding to the G protein-coupled receptors expressed exclusively on granulosa cells (GCs) (17,18). An adenylate cyclase-mediated signal is activated, followed by the expression of multiple mRNAs that encode proteins responsible for cell proliferation, differentiation, and function. FSH stimulates GC proliferation and growth (mitogenic action) and induces aromatase activity via P450 activation (19). Concomitantly, the number of FSH receptors increases as GCs respond to FSH. The regulation of GC FSH receptor activity involves not only a direct cAMP-mediated FSH influence on its own receptor gene but also estrogen and other inhibitory agents, including epidermal growth factor, fibroblast growth factor, and GnRH-like protein. Inhibin and activin, which are also produced by granulosa cells in response to FSH, have autocrine activity and stimulate FSH receptor production, thus enhancing FSH action (19,20).

### Luteinizing hormone

The LH beta subunit is comprised of 121 AAs, which is a difference that confers specific biologic activity and facilitates its interaction with the LH receptor (3). LH  $\beta$ -subunits contain a single site with N-linked glycosylation (Asn 30)

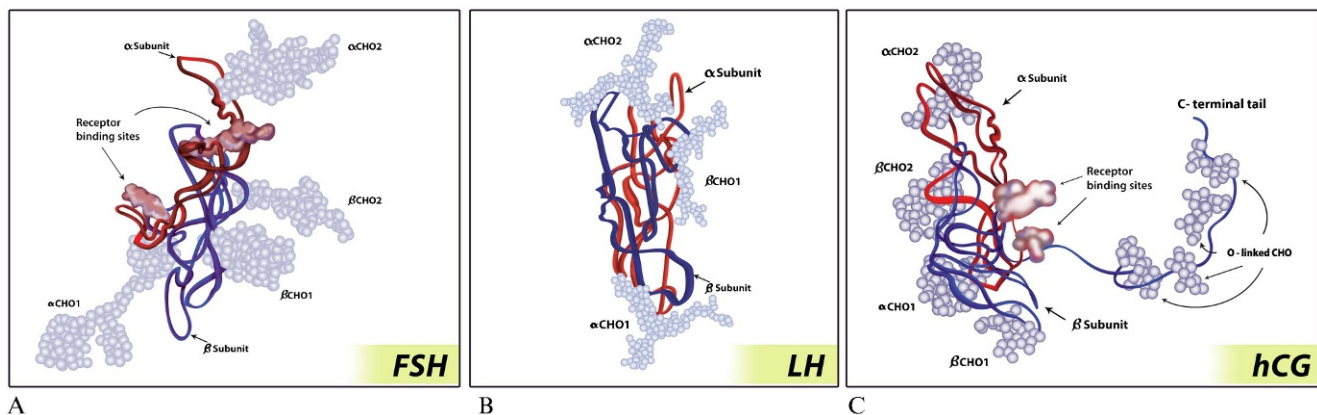
and fewer sialic acid residues (only 1 or 2); as such, LH has a short half-life of only 20 to 30 minutes (Figure 1) (15).

LH plays a key role in promoting steroidogenesis and developing the leading follicle; it has different functions at different stages of the cycle (19-22). During the early follicular phase, LH stimulates androgen production by theca cells. Cholesterol is converted into androgens (testosterone and androstenedione) through the transcription of the cholesterol side-chain cleavage enzyme (P450<sub>sc</sub>), P450<sub>c17</sub>, and  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ -HSD) genes (Figure 2). Androgens are then transferred to the GC and transformed into estrogens via aromatization (21). Finally, LH promotes final follicular maturation via its direct effects on the GC in the late follicular phase (22). Theca cells and GCs also secrete peptides, including insulin-like growth factor (IGF), inhibin, and activin, which act as both autocrine and paracrine factors and modulate LH-mediated androgen production in the thecal compartment as well as FSH-mediated aromatization in GCs (19,20,23).

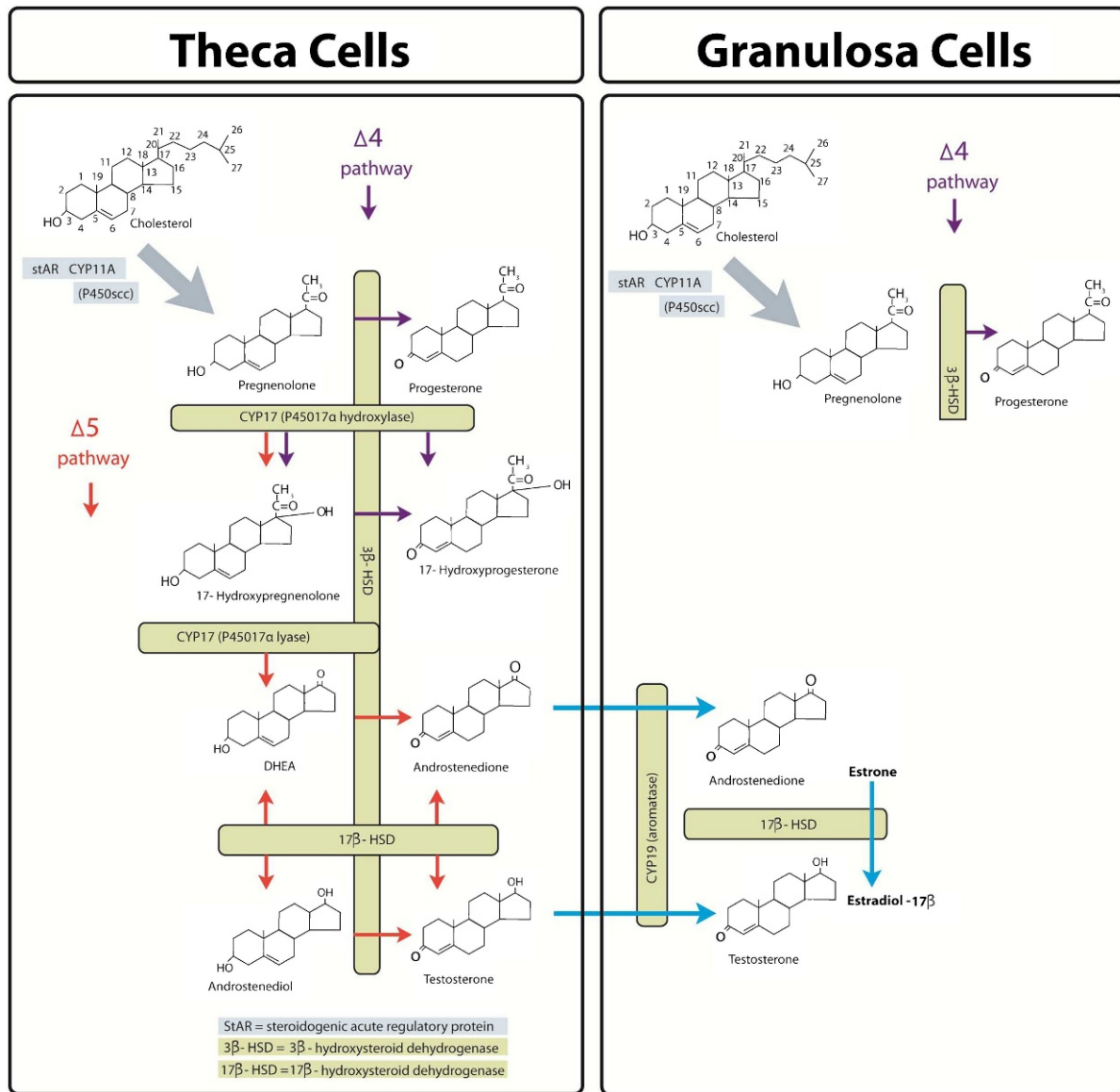
### Human chorionic gonadotropin

Although the beta subunit of hCG has an AA sequence similar to LH, a notable difference is the presence of a long carboxyterminal segment with 24 AAs containing four O-linked oligosaccharide sites (Figure 1) (3,9). In addition, hCG beta subunits contain two sites of N-linked glycosylation compared with a single LH site. Due to the higher number of both glycosylation sites and sialic acid residues (approximately 20) compared with LH, hCG exhibits a markedly longer terminal half-life of 24 hours compared with approximately 30 minutes for LH (15).

Due to their similar structure, hCG binds the same receptor as LH. In gonadotropin therapy, hCG is used to promote the final follicular maturation stages and progression of the immature oocyte at prophase I (the germinal vesicle stage) through meiotic maturation to metaphase II (3). The meiotic process requires approximately 36 hours to complete; a few hours later, ovulation occurs. As such, follicular aspiration upon oocyte retrieval is timed with hCG administration in IVF. In addition, hCG can be used to



**Figure 1 - Gonadotropin Molecules.** The alpha and beta subunits are represented by red and blue strands, respectively, whereas the carbohydrate chains are represented by light blue balls. **A) Follicle-stimulating hormone.** FSH is a glycoprotein composed of two subunits, the alpha subunit (red) and the beta subunit (blue). There are four carbohydrate attachment sites, two in each subunit. **B) Luteinizing hormone.** LH is a glycoprotein with two subunits, the alpha subunit (red), which is similar to FSH and hCG, with two carbohydrate attachment sites, and the beta subunit (blue) with only one carbohydrate attachment site. **C) Human chorionic gonadotropin.** hCG has structural attributes similar to LH. A notable exception is the presence of a long carboxy-terminal segment that is O-glycosylated (O-linked CHO), conferring a longer half-life to hCG.



**Figure 2 - Human Steroidogenesis.** The starting point for steroid biosynthesis is the conversion of cholesterol in pregnenolone by P450scc. One route for pregnenolone metabolism is the delta-5 pathway (red arrows) through CYP17 (P450c17). Pregnenolone hydroxylation at the C17a position forms 17-hydroxypregnenolone, and subsequent removal of the acetyl group forms the androgen precursor dehydroepiandrosterone (DHEA). An additional route for pregnenolone metabolism is the delta-4 pathway (purple arrows), in which pregnenolone is converted to progesterone by 3b-HSD (an irreversible conversion). Progesterone is then converted to 17-hydroxyprogesterone by CYP17. In humans, 17-hydroxyprogesterone cannot be further metabolized. Importantly, CYP17 is exclusively located in theca and interstitial cells in the ovary extrafollicular compartment, whereas CYP19 (aromatase), which converts androgens to estrogens, is expressed exclusively in GCs, which are in the intrafollicular compartment. Androgen aromatization to estrogens is a distinct activity that occurs in the granulosa layer, and it is induced by FSH via P450 aromatase (P450arom) gene activation.

maintain luteal function until placental steroidogenesis is well established (19).

**■ MILESTONES IN GONADOTROPIN DEVELOPMENT**

Researchers began to develop gonadotropin preparations in 1910, when experimental evidence suggested that the pituitary has a role in regulating gonadal stems (2). Zondek, in collaboration with Ascheim (1927), demonstrated that blood and urine from pregnant women contained a gonad-stimulating substance capable of inducing both follicular

maturation and ovarian stromal luteinization when injected into immature mice. This substance was shown to be hCG, which is produced in the placental tissue of the syncytiotrophoblast (2,3,24). *In vitro* hCG production was then possible through placental tissue culture, and commercial hCG was first available in 1931 (2,3). Early observations revealed that hCG administered alone in the follicular phase failed to promote follicular development and ovulation, thus indicating that hCG had no effect in the absence of FSH (2,25). In contrast, a number of trials demonstrated that gonadotropins extracted from the blood of pregnant mares (PMSG) and from humans (post-mortem pituitary glands)



induced an ovarian response, but attempts to fully induce ovulation produced inconsistent results (3,26). Human pituitary extracts and PMSG were used in both Europe and the United States until the early 1960s, despite findings that such treatments produced neutralizing antibodies (anti-hormones) that rendered the ovaries unresponsive to repeated stimulation (2,3,27). By the mid-1980s, cases of dementia and death due to iatrogenic Creutzfeldt-Jacob disease (CJD) were identified in Australia, France, and the United Kingdom and linked to human pituitary gonadotropin (hPG) use. As a consequence, hPG was banned from the market approximately 20 years after its introduction (2,3).

The recognition that animal gonadotropins induce anti-hormone antibody production, which neutralized not only the preparation administered but also endogenous gonadotropins, was the driving force behind gonadotropin extraction and purification from human sources. In the 1940s, researchers began extracting gonadotropins from urine: hCG in 1940 and human menopausal gonadotropin (hMG) in 1949. A decade later, the first urinary forms of hCG and hMG became commercially available (2,3). Further improvements in the purification methods produced FSH-only products in the 1980s and highly purified urinary FSH (HP-hFSH) in 1993 (2,3). Advances in DNA technology enabled the development of recombinant human FSH (rec-hFSH), which became commercially available in 1995 (2,3,28). In 2000, recombinant human LH (rec-hLH) became available and, with the launch of recombinant hCG (rec-hCG) in 2001, the full recombinant gonadotropin portfolio was launched (2,3). The most recent developments include

the introduction of the filled-by-mass (FbM) rec-hFSH formulation in 2004, which improved batch-to-batch consistency compared with products quantified by the standard rat *in vivo* bioassay; long-acting FSH gonadotropin in 2010; and novel pen injector devices to deliver precise recombinant FSH, LH, and hCG doses in 2011 (29-35) (Figure 3).

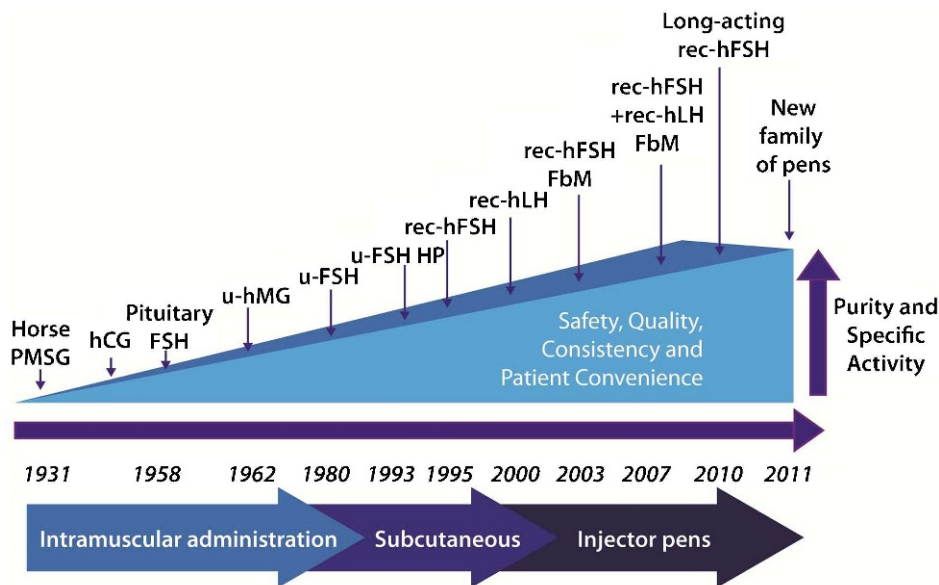
### ■ PREPARATIONS CURRENTLY AVAILABLE FOR CLINICAL USE

#### Human menopausal gonadotropin (menotropin)

Menotropin is extracted from the urine of postmenopausal women (2). Early preparations contained varying levels of FSH, LH, and hCG in only 5% pure forms (3). The purification techniques were improved, resulting in FSH and LH with activities standardized at 75 IU for each type of gonadotropin, as measured using a standard *in vivo* bioassays (Steelman-Pohley assay). hMG preparations have both FSH and LH activity, but the latter is primarily derived from the hCG component in postmenopausal urine, which is concentrated during purification (2,36,37). Occasionally, hCG is added to induce a desired level of LH-like biological activity (2). In 1999, purified hMG gonadotropins were introduced, which facilitated its subcutaneous (SC) administration (3,29). Currently, both conventional hMG and highly purified hMG (HP-hMG) are commercially available at an FSH:LH ratio of 1 : 1 (29).

#### Urinary FSH (urofollitropin)

Urinary FSH preparations are produced by removing LH with polyclonal antibodies. The production process is



**Figure 3 - Milestones in the Development of Gonadotropin Preparations.** FSH was originally derived from animal (pregnant mare serum) or human (post-mortem pituitary glands) sources, but these preparations were abandoned due to safety concerns. Gonadotropins were first extracted from urine in the 1940s; human chorionic gonadotropin (hCG) in 1940; and then human menopausal gonadotropin (hMG) in 1949. Over a decade later, the first urinary forms of hCG and hMG became commercially available. Further improvements in purification methods yielded follicle-stimulating hormone (FSH)-only products in the 1980s and the subsequent development of highly purified FSH (HP-hFSH), which became available 10 years later, in 1993, and allows for subcutaneous injection. In the 1970s and 1980s, advances in DNA technology enabled the development of recombinant human FSH (rec-hFSH), which became commercially available in 1995. In 2000, recombinant human luteinizing hormone (rec-hLH) became available, and with the launch of recombinant human hCG (rec-hCG) in 2001, the full recombinant gonadotropin portfolio was available. The most recent developments include the filled-by-mass (FbM) follitropin alfa formulation, the fixed combination of follitropin alfa + lutropin alfa, long-acting FSH gonadotropin, and a new family of prefilled pen injector devices.



passive because LH is separated from the bulk material, and FSH, together with certain other urinary proteins, is collected and lyophilized. Though they were biologically more pure, early preparations still contained high levels of other urinary proteins (38). Further technological advances facilitated the use of highly specific monoclonal antibodies to extract FSH and produce highly purified FSH (HP-hFSH). The latter has been commercially available since 1993 and contains <0.1 IU of LH and <5% of unidentified urinary proteins. The specific activity of FSH is approximately 10,000 IU/mg protein, whereas that of the earlier urinary hMG preparations was 100–150 IU/mg protein (Table 1). Similar to HP-hMG, the enhanced purity of HP-hFSH enabled SC delivery (3). SC gonadotropin administration represented an important advance for patients. Consistently better tolerability (decreased pain at the injection site) was reported for SC injections compared with the intramuscular route (39,40). Moreover, SC administration allows self-administration, which is more convenient and less time consuming because patients require fewer visits to the clinic or hospital for injections (39,40).

### Recombinant FSH

Recombinant technology has met the need for a more reliable FSH source. Under appropriate conditions, the genes that code for the human FSH alpha and beta subunits are incorporated into nuclear DNA of a host cell via a plasmid vector using spliced DNA strings containing the FSH gene and bacterial DNA segments (2,3,41). Early recombinant technology used *Escherichia coli*. However, due to the complex human gonadotropin structure and the need for post-translational glycosylation, which defines the degradation time and bioactivity, all recombinant gonadotropins are now produced using the Chinese hamster ovary (CHO) cell line. These cells are genetically stable, fully characterized, and easily transfected with foreign DNA. Furthermore, the cells can be grown in cell cultures on a large scale, and can produce adequate levels of biologically active recombinant gonadotropins (2,41).

Two types of recombinant FSH (rec-hFSH), the alfa and beta follitropins, are available for clinical use (2). In follitropin alfa, two separate vectors, one for each subunit, are used to construct the master cell bank for an FSH-producing cell line. Follitropin beta uses a single vector that contains the coding sequences for both subunit genes (41,42). The subsequent production steps are similar for both preparations. Nevertheless, in addition to a series of anion and cation exchange chromatography steps, hydrophobic chromatography and size exclusion chromatography

used to produce follitropin beta, an immunoaffinity step with a specific monoclonal antibody similar to the antibody used for HP-hFSH production is used for follitropin alfa (Figure 4) (2,41). Due to the slight differences in their production and purification procedures, the preparations are not identical, with variations in posttranslational glycosylation that yield different sialic acid residue compositions and different isoelectric coefficients (3,43,44). While follitropins alfa and beta are similar to the native FSH isoforms in the blood around mid-cycle (more basic isoforms), they differ slightly in charge heterogeneity, as follitropin alfa has slightly more acidic glycoforms than follitropin beta (43,45). The preparations include equivalent immunopotency, *in vitro* biopotency, and internal carbohydrate complexity (43,46). The initial and terminal half-lives after the administration of 150 IU recombinant FSH are 2 and 17 hours, respectively. Given their intrinsically similar structures, clinical efficacy is expected to be the same (3,43,46).

### Long-acting recombinant FSH (corifollitropin alfa)

Due to the relatively short half-life of FSH, daily FSH injections are used to prevent serum FSH levels from decreasing below the threshold that causes follicular growth arrest (47). After each injection, the peak serum FSH levels are reached within 10–12 hours; the FSH levels then decline until the next injection. Steady state levels are reached only after treatment for 3–5 days; thus, dose adjustments before day 5 of stimulation are not advised (48).

Recently, a novel long-acting gonadotropin molecule was developed by combining rec-hFSH with the hCG C-terminal peptide (CTP) using site-directed mutagenesis and gene transfer techniques (48). Its longer half-life is due to the hCG CTP, which includes four additional O-linked carbohydrate side chains, each with two terminal sialic acid residues (15,49,50). The new molecule was created using a chimeric gene containing the sequence that encodes CTP fused to the translated human FSH beta subunit sequence. The chimera was then transfected with the common glycoprotein alpha subunit and expressed in CHO cells. The CTP sequence does not significantly affect assembly or secretion of the intact dimer by stable cell lines. The chimeric recombinant molecule has similar *in vitro* receptor binding and steroidogenic activity compared with wild-type FSH but exhibits significant enhancement of its *in vivo* activity and plasma half-life (48,51).

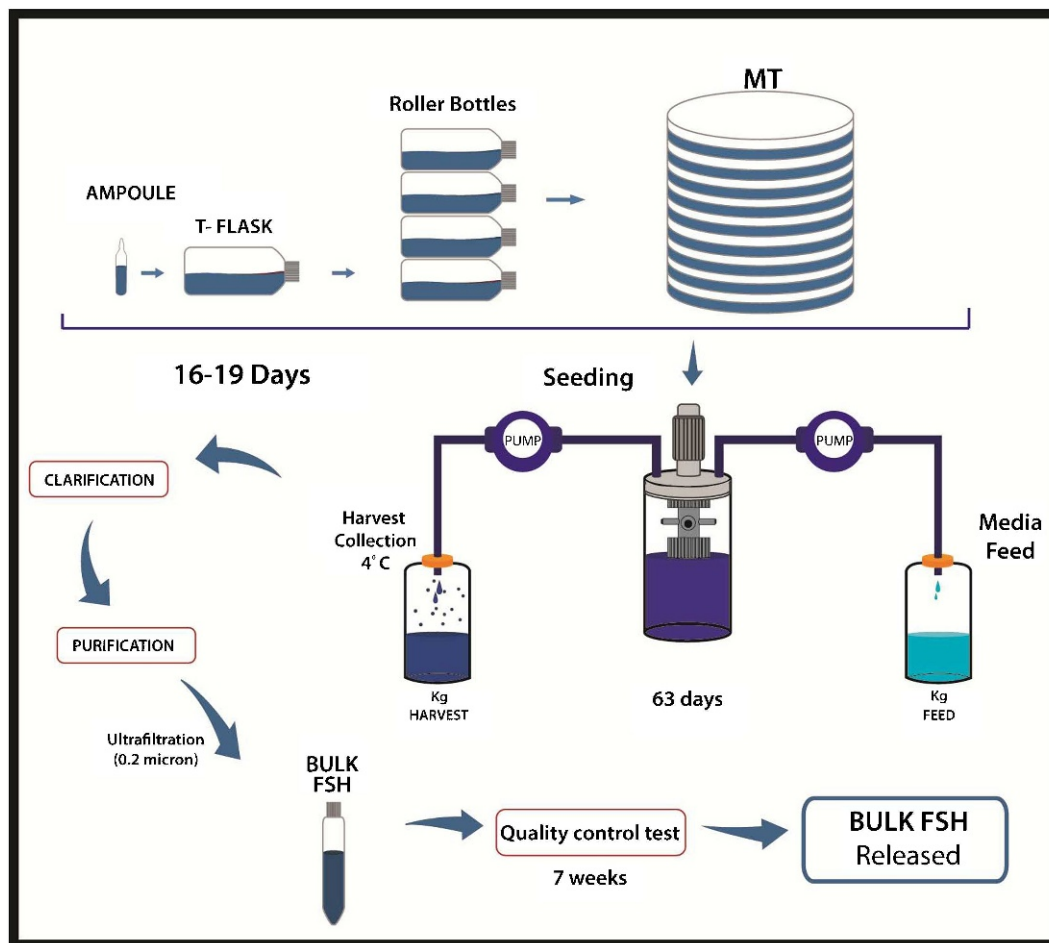
Corifollitropin alfa, initially produced in 2010, exclusively interacts with FSH receptors and has a plasma half-life of 65 hours (33,51). Clinical studies indicate that a single

**Table 1 - Differences between gonadotropin formulations.**

	Purity (gonadotropin content)	Mean Specific Activity (IU/mg protein)	LH Activity (IU/vial)	Injected Protein per 75 IU (mcg)
hMG	<5%	~100	75*	~750
HP-hMG	<70%	2,000-2,500	75*	~33
rec-hFSH				
Follitropin beta	>99%	7,000-10,000	0	8.1
Follitropin alfa	>99%	13,645	0	6.1
Lutropin alfa (rec-hLH)	>99%	22,000	75	3.7

rec-hFSH: recombinant human follicle-stimulating hormone; hMG: human menopausal gonadotropin; HP-hMG: highly purified human menopausal gonadotropin.

\*Primarily derived from the hCG component, which is preferentially concentrated during the purification process and may be added to generate the desired level of LH-like biological activity (approximately 8 IU of hCG per vial of 75 IU).



**Figure 4** - Recombinant gonadotropin technology. Chinese hamster ovary cells are first grown in T-flasks, then subcultured in roller bottles and allowed to expand for up to 36 days. Next, the cells are mixed with a microcarrier bead suspension and transferred to a bioreactor vessel continuously perfused with a growth-promoting medium for an average of 34 days. The cell culture supernatant medium containing ‘crude glycoprotein’ is collected from the bioreactor and stored at 48°C until purification. The protein is purified by chromatography followed by ultrafiltration. The final product is released after extensive quality control testing over 7 weeks.

injection of corifollitropin alfa can replace the first seven daily standard gonadotropin injections and that stimulation could be continued with daily FSH injections until the final oocyte maturation had been reached (52).

### Gonadotropin preparations with LH activity

Currently, three groups of commercially available gonadotropin preparations contain LH activity: (i) urinary hMG, in which LH activity depends on hCG rather than pure LH glycoprotein; (ii) pure LH glycoprotein produced by recombinant technology (lutropin alfa), and (iii) a combination of pure FSH and LH glycoproteins in a fixed ratio of 2:1, which is also manufactured through recombinant technology (Table 1) (3).

While hMG has been used for ovarian stimulation since 1960, lutropin alfa was introduced in 2000 for women with gonadotropin insufficiency. It is intended to be administered through subcutaneous daily injections. Recently, a new prefilled pen device was introduced for rec-hLH administration (53). Currently, rec-hLH is used both to support follicular development during COS in hypogonadotropic hypogonadal women and to offer LH supplementation to subsets of

women undergoing COS (2,54,55). Rec-hLH has three major differences compared to hMG. First, it has higher purity and specific activity due to the use of recombinant technology. Second, it is associated with better dose precision due to the vial/device filling method, which virtually eliminates batch-to-batch variation (2,30,56). Third, the LH activity is derived directly from pure LH glycoprotein, unlike hMG, in which hCG is concentrated during purification or added to achieve the desired level of LH-like biological activity (2). LH and hCG differ in their carbohydrate moiety compositions, which in turn, affect bioactivity and half-life. In serum, LH activity is 30-fold higher when hCG is used because it binds LH receptors with greater affinity. Rec-hLH is eliminated with a terminal half-life of 10-12 hours, in contrast to the 24-31 hours required for hMG preparations with hCG-driven LH activity (15,57-61).

A new combination of rec-hFSH and rec-hLH (follitropin alfa + lutropin alfa) at a 2:1 ratio was launched in 2007. This combination is advantageous for women who require LH supplementation because a single injection, rather than two, is used to deliver both preparations. The bioequivalence of



rec-hFSH and rec-hLH administered alone or in combination is similar (32,62).

### Human chorionic gonadotropin

hCG administration is the gold standard for promoting ovulation induction as a substitute for the mid-cycle LH surge (63). Due to structural and biological similarities, hCG and LH bind to and activate the same receptor (64). However, the luteotropic activity of hCG is markedly higher than that of LH due to its longer half-life and greater receptor affinity (57,65). Currently, urinary hCG preparations are marketed in lyophilized vials with 5,000 or 10,000 IU for intramuscular use. In 2001, an hCG preparation (choriogonadotropin alfa) was launched using recombinant technology. Recombinant hCG (rec-hCG) is available in prefilled syringes containing 250 mcg of pure hCG, which is equivalent to approximately 6,750 IU of urinary hCG (3). Due to its higher purity, rec-hCG is better tolerated and used subcutaneously, thus allowing patient self-administration (66). Nevertheless, the clinical efficacy of both urinary and recombinant preparations does not seem to differ (67). In a Cochrane meta-analysis including 11 randomized controlled trials (RCTs) of IVF with 1,187 women, Youssef et al. compared rec-hCG vs. urinary hCG for triggering final oocyte maturation. A significant difference was not detected in the main outcome measurements between the drugs: ongoing pregnancy/live birth rate (6 RCTs: odds ratio [OR]=1.04, 95% confidence interval [CI]: 0.79 to 1.37; I<sup>2</sup>=0%), incidence of ovarian hyperstimulation syndrome (OHSS) (3 RCTs: OR=1.5, 95% CI: 0.37 to 4.1; I<sup>2</sup>=0%) and the number of retrieved oocytes (9 RCTs: Mean difference=-0.04, 95% CI: -0.69 to 0.62; I<sup>2</sup>=18%) (67).

Table 2 summarizes the gonadotropin preparations currently available for clinical use.

### ■ QUALITY AND SAFETY PROFILES

Manufacturing urine-derived gonadotropins requires high levels of human urine as a primary source. In the 1960s and 1970s, when the demand for gonadotropins was low, the source material quality was controlled. However, the widespread availability of infertility treatments has rapidly increased demand since 1980. Unlike blood, human urine is not subject to specific regulations regarding collection. Because collected urine is pooled, the donor source cannot be traced, and quality cannot be checked throughout all manufacturing steps (38,68,69). However, extraneous urinary proteins may account for more than 30% of the protein levels in highly purified hMG products even with sophisticated purification techniques (56,69). Certain impurities have been identified as prion proteins, which have been associated with transmissible spongiform encephalopathy (TSE) diseases (70). Prion inactivation in urine-derived material may also denature other proteins, including FSH. In fact, several regulatory agencies limit urine-derived products (38,68). In contrast, each product batch of recombinant gonadotropin is routinely characterized and controlled using physicochemical techniques. The techniques include size exclusion high-performance liquid chromatography (SE-HPLC), which facilitates assessment of both the integrity and the levels of glycoproteins, as well as isoelectric focusing (IEF) and glycan mapping, which are used to characterize the protein glycoforms in each preparation (71,72). Due to the linear relationship between recombinant gonadotropin mass and biological activity, a new method was developed to calibrate each batch of follitropin alfa, lutropin alfa, and choriogonadotropin. Although the Steelman–Pohley assay is used to quantify the protein levels in urinary preparations, which inherently vary up to 20%, recombinant products are filled and released based on protein mass (FbM) (30,73). FSH at

**Table 2 - The most common gonadotropins available for clinical use.**

Product	Technology	Brand name	Manufacturer
HMG	Urine derived	Menogon; Repronex	Ferring
HP-hMG	Urine derived	Menopur	Ferring
HP-hFSH	Urine derived	Merional	IBSA
		Fostimon	IBSA
U-hCG	Urine derived	Bravelle	Ferring
		Choragon	Ferring
Rec-hFSH	Recombinant	Brevactid	Ferring
		Choriomon, Gonasi HP	IBSA
		APL	Wyeth-Ayerst
		Biogonadyl	Biomed-Lublin
		Primogonyl	Schering-Plough
		Endocorion	Win-Medicare
		Corion	Wyeth-Ayerst
Follitropin beta	Recombinant	Puregon; Follistim	Merck Sharp & Dohme
Follitropin alfa	Recombinant	GONAL-f	MerckSerono
Long-acting FSH	Recombinant	Elonva	Merck Sharp & Dohme
Corifollitropin alfa			
Rec-hLH	Recombinant	Luveris	MerckSerono
Lutropin alfa			
Rec-hFSH + rec-hLH 2:1	Recombinant	Pergoveris	MerckSerono
Follitropin + Lutropin alfa			
Rec-hCG	Recombinant	Ovidrel; Ovitrelle; Ovidrelle	MerckSerono

HMG: human menopausal gonadotropin; HP-hMG: highly purified human menopausal gonadotropin; u-hCG: urinary human chorionic gonadotropin; rec-hFSH: recombinant human follicle-stimulating hormone; rec-hLH: recombinant human luteinizing hormone; rec-hCG: recombinant human chorionic gonadotropin.



75 IU was assessed using the Steelman-Pohley method, which corresponds to 5.0-5.5 µg of follitropin alfa, with a dose variability of only 2% (3,30). This method ensures that a precise dose is delivered, thus maximizing the beneficial effects of gonadotropin therapy (3,29).

The pharmaceutical presentation of urinary gonadotropins consists of a freeze-dried lyospheres containing either 75 IU of FSH/hMG or 5,000/10,000 IU of hCG. The lyophilized powder is then reconstituted using sterile water before injection (29). Higher gonadotropin purity yields higher specific activity, and therefore, less material is injected for the desired effect. Through these characteristics, highly purified urine-derived and recombinant gonadotropins can be administered subcutaneously (56). Given the high specific activity of recombinant gonadotropins, minimal volumes are injected, and injection devices have been developed to deliver the drug (40). The first injector was an adapted insulin pen. Early studies showed that drug delivery was more precise and better tolerated using the pens than syringe injections. Due to unavoidable losses during syringe filling and/or removing excess air, 18% of the FSH amount is lost in conventional syringe applications when compared with a ready-for-use solution in a pen device (40). Recently, novel devices were specifically developed for gonadotropin administration. The first generation was released in 2004 followed by a second generation in 2011 (34,35). They are presented as ready-to-use, compact, and disposable pens, FbM with a fixed drug dose that can be administered in fractions over several days (30,56). In an RCT including 100 women, the efficacy, convenience, and local reactions after follitropin alfa administration were compared following the use of either the pen device or a conventional syringe. Outcomes, including self-administration and patient satisfaction ( $p < 0.001$ ), the overall incidence of local reactions ( $p = 0.04$ ), the overall pain score ( $p < 0.001$ ), and burning sensation at the injection site ( $p = 0.04$ ), clearly favored the pen device group (74). Later, in 2007, patients and their partners received nurse-led training on three gonadotropin presentations: (i) powdered urofollitropin administered using conventional needles and syringes, (ii) follitropin beta in a premixed and prefilled cartridge with a reusable injection device, and (iii) follitropin alfa in a disposable, premixed, and prefilled injection device. One hundred twenty-three participants attended the training and were asked to complete a post-training questionnaire. More participants expressed a preference for using pen injectors compared with conventional syringes (84.6% vs. 5.7%;  $p < 0.0001$ ). Of the 94 participants who preferred a particular device, more preferred the follitropin alfa prefilled pen (68.1%) than either the follitropin beta cartridge and pen (24.5%;  $p < 0.0001$ ) or urofollitropin with a needle-free reconstitution device and conventional syringe (7.4%;  $p < 0.0001$ ) (75). In conclusion, recombinant technology, the FbM method, and the use of pen devices for gonadotropin administration represent important advancements that have made infertility treatments more patient friendly (40,74,76).

## ■ CLINICAL EFFICACY OF GONADOTROPINS

The literature is rich in meta-analyses comparing efficacy for different gonadotropin products (77-81). The most recent studies are summarized in Table 3. Despite the heterogeneity of several of these meta-analyses pertaining the

different stimulation protocols and choice of fertilization with standard *in vitro* fertilization or intracytoplasmic sperm injection (ICSI), the overall conclusion is that both urinary gonadotropins, mainly hMG preparations, and recombinant FSH have similar efficacy in terms of achieving a pregnancy or live birth per treatment cycle. While some of these studies were in favour of hMG preparations, albeit the lower confidence limits were 1% or less, others reported no differences in pregnancy outcomes between the two treatments. Furthermore, no significant differences were noted for spontaneous abortion, multiple pregnancy, cycle cancellation and OHSS rates. Notably, these studies did not stratify patients according to the need for LH supplementation during COS. This is a relevant aspect given the fact rec-hFSH has solely FSH activity and recent evidence indicates that a subset of women benefit from LH supplementation during COS (55).

Several studies have also compared the potency of different gonadotropin formulations (29,60,82,83). In an RCT involving 629 women undergoing IVF/ICSI treatment with pituitary down-regulation, Hompes et al. compared HP-hMG and rec-hFSH. In their study, more oocytes were retrieved from patients treated with rec-hFSH (7.8 and 10.6, respectively;  $p < 0.001$ ), with no differences in pregnancy rates (82). Similarly, in an RCT involving 280 women undergoing IVF/ICSI with GnRH antagonists, Bosch et al. obtained more oocytes from patients who received rec-hFSH compared with those who received hMG ( $14.4 \pm 8.1$  vs.  $11.3 \pm 6.0$ , respectively;  $p < 0.001$ ). No significant differences were observed in the ongoing pregnancy rate per initiated cycle (35.0 vs. 32.1%, respectively; RR = 1.09; 95% CI: 0.78-1.51; risk difference [RD] = 2.9%) (60). Recently, in a large RCT involving more than 700 patients in a single blastocyst transfer IVF program, Devroey et al. confirmed that rec-hFSH results in more oocytes than HP-hMG when used at the same doses ( $10.6 \pm 5.8$  vs.  $9.1 \pm 5.2$ ;  $p < 0.001$ ) (83). We also compared different gonadotropin products in a large observational study involving 865 women undergoing IVF/ICSI with pituitary down-regulation, and found that the total gonadotropin dose was significantly lower in women who received rec-hFSH ( $2,268 \pm 747$  IU) compared with hMG ( $2,685 \pm 720$  IU) or HP-hMG ( $2,903 \pm 867$  IU;  $p < 0.001$ ). In our study, the live birth rates per cycle initiated were the same in patients who received rec-hFSH (34.7%), hMG (35.5%), or HP-hMG (40%). However, the total gonadotropin dose required for a live birth was lower when rec-hFSH was compared with hMG (52% reduction) and HP-hMG (21% reduction) (29). The available data support the notion that recombinant FSH is more potent than hMG during COS (29,60,82,83).

The clinical efficacy of gonadotropins has also been assessed with regard to the vial-filling method. In a meta-analysis including four RCTs involving 1,055 women and two case-control studies with 272 patients undergoing IVF, the average rec-hFSH dose per patient was 230 IU lower when the drug was FbM compared with the filled-by-bioassay method (weighted mean difference [WMD] = -230.3; 95% CI: -326 to -134.5;  $p < 0.001$ ). In addition, the number of treatment days was reduced by 0.48 (WMD = -0.48; 95% CI: -0.69 to -0.27,  $p < 0.001$ ), whereas the numbers of oocytes retrieved (WMD = 0.84; 95% CI: 0.18 to 1.51;  $p < 0.01$ ) and embryos developed (WMD = 0.88; 95% CI: 0.40 to 1.37;  $p < 0.001$ ) were higher in patients who received FbM formulations. The clinical pregnancy (OR = 1.3; 95% CI: 0.91



**Table 3** - Meta-analyses comparing urinary and recombinant gonadotropins for controlled ovarian stimulation in *in vitro* fertilization.

Authors, Year	Gonadotropins	No. RCT	No. Patients	Main Findings
Coomarasamy et al., 2008	rec-hFSH; hMG	7	2,159	Higher clinical pregnancy (RR = 1.17, 95% CI: 1.03-1.34) and live birth rates (RR = 1.18, 95% CI: 1.02-1.38; $p=0.03$ ) with hMG. No significant differences in the spontaneous abortion, multiple pregnancy, cycle cancellation, or OHSS rates.
Al Inany et al., 2009	rec-hFSH; hMG; HP-hMG	6	2,371	Overall, no significant differences in the clinical, ongoing pregnancy, or live birth rates. Higher ongoing pregnancy/live-birth rates with HP-hMG (OR = 1.31, 95% CI: 1.02-1.68; $p=0.03$ ) after grouping the treatment cycles by method, ICSI and IVF.
Jee et al., 2010	rec-hFSH; HP-hMG	5	2,299	No difference in ongoing pregnancy rate per initiated cycle (RR = 1.10; 95% CI: 0.96-1.26) or live birth rates per embryo transfer (RR = 1.14; 95% CI: 0.98-1.33).
Van Wely et al., 2010	rec-hFSH; hFSH-P; HP-hFSH; hMG; HP-hMG	28	7,339	Overall, no difference in live birth or OHSS rates.
Van Wely et al., 2012	rec-hFSH; hMG; HP-hMG	12	3,197	Fewer clinical pregnancies (OR = 0.85; 95% CI: 0.74-0.99; $I^2=0\%$ ; $p=0.03$ ) and live births with rec-hFSH (OR = 0.84; 95% CI: 0.72-0.99; $I^2=0\%$ ; $p=0.04$ ).
Gerli et al., 2013	rec-hFSH; hFSH-P; HP-hFSH	8	955	No difference in the clinical pregnancy (OR = 0.85, 95% CI: 0.68 to 1.07) or live birth rates (OR = 0.84; 95% CI: 0.63-1.11).

RCT: randomized controlled trial.

rec-hFSH: recombinant human follicle-stimulating hormone; hMG: human menopausal gonadotropin; HP-hMG: highly purified human menopausal gonadotropin; hFSH-P: purified urinary follicle-stimulating hormone; HP-hFSH: highly purified urinary follicle-stimulating hormone; COS: controlled ovarian stimulation.

RR: relative risk; CI: confidence interval; OR: odds ratio;

IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection; OHSS: ovarian hyperstimulation syndrome.

to 1.82) and ovarian hyperstimulation syndrome (OHSS) incidence rates (OR = 0.78; 95% CI: 0.45 to 1.36) were the same for both formulations (84).

Similarly, long-acting and daily-use recombinant FSH preparations have been compared. A meta-analysis of four pharmaceutical industry-sponsored RCTs that included 2,377 participants evaluated the effectiveness, safety, and tolerability of corifollitropin alfa compared with follitropin beta in IVF/ICSI cycles with GnRH antagonists. The results favored corifollitropin alfa with regard to the number of oocytes retrieved (WMD = 1.99; 95% CI: 1.02 to 2.97;  $p < 0.0001$ ), the number of mature oocytes (WMD = 1.92; 95% CI: 1.25 to 2.59;  $p < 0.001$ ), and the number of embryos formed (WMD = 1.09; 95% CI: 0.68 to 1.49;  $p < 0.0001$ ). The clinical pregnancy, live-birth, and miscarriage rates were similar regardless of the drug used for COS. The median duration of stimulation was 9 days in both groups, indicating that two additional single daily rec-hFSH injections are required to complete the treatment regimen with corifollitropin alfa. Notably, corifollitropin alfa resulted in higher cycle cancellations due to an excessive response (OR = 5.67; 95% CI: 1.07 to 30.13;  $p = 0.04$ ), but the OHSS incidence was not significantly different between the groups (OR = 1.29; 95% CI: 0.78 to 2.26) (85). These results were further corroborated by a recent Cochrane review of the same studies (86). The main shortcoming of corifollitropin alfa is that the dose cannot be adjusted during ovarian stimulation, which is a particularly relevant limitation for patients at risk of developing OHSS. From the data available, corifollitropin alfa is likely efficacious and safe for COS in normal responders, but it is not recommended for women at risk of OHSS, such as women with polycystic ovaries (85).

#### Luteinizing hormone supplementation during COS

The "LH window" concept outlined by Shoham in 2002 proposes that without a threshold level of serum LH, estradiol production is insufficient for follicular development, endometrial proliferation, and corpus luteum formation. However, exposing the developing follicle to excessive

LH would suppress GC proliferation, induce follicular atresia of non-dominant follicles and premature luteinization, and impair oocyte development (87). Under this concept, optimal follicular development occurs when LH is above a threshold of 1.1 and below a ceiling of 5.1 IU/L (87,88). The validity of the LH threshold hypothesis has been demonstrated in patients with hypogonadotropic hypogonadism. These women do not achieve adequate steroidogenesis unless LH is added to the stimulation regimen (88).

Unlike pituitary insufficiency, most women undergoing COS for IVF have adequate endogenous LH levels and thus do not require LH supplementation (Table 4) (89-92). Indeed, only 1% of LH receptors must be occupied to drive adequate ovarian steroidogenesis (93,94). Nevertheless, the ovarian response to FSH-only gonadotropins is suboptimal in certain patient groups, including older women ( $\geq 35$  years old) (55,95) and women with a diminished ovarian reserve (54,81) or highly suppressed endogenous LH (96-100). Further, a subset of normogonadotropic women have a suboptimal response to FSH stimulation despite a normal ovarian reserve (101-105). All these patients share a similar trait, less sensitive ovaries, which can be explained by several factors, including reduced paracrine ovarian activity (106), LH receptor polymorphisms (105), reduced androgen secretory capacity (107), fewer functional LH receptors (108), and reduced LH bioactivity despite normal LH immunoreactivity (109-110).

It has been hypothesized that these women would benefit from preparations containing LH, which would act at the follicular level. An increase in androgen production for future aromatization into estrogens could restore the follicular milieu and thus positively impact oocyte quality (97,99,103,111,112). In fact, several studies have assessed the utility of LH supplementation during COS (Table 4). A recent meta-analysis by Hill et al., which included seven RCTs and 902 older women undergoing COS for IVF, demonstrated significantly higher embryo implantation (OR = 1.36; 95% CI: 1.05 to 1.78,  $I^2 = 12\%$ ) and clinical pregnancy rates (OR = 1.37; 95% CI: 1.03 to 1.83,  $I^2 = 28\%$ )



**Table 4 - Meta-analyses comparing controlled ovarian stimulation with and without recombinant LH supplementation in *in vitro* fertilization.**

Author; Year	Patient Inclusion Criteria	GnRH analogue	No. RCT	No. Patients	Primary Outcomes; (OR; 95% CI)	Secondary Outcomes
Mochtar et al., 2007	Unselected	Agonist	11	2,396	No differences in CPR <sup>1</sup> (1.15; 0.91-1.45) or OPR <sup>1</sup> (1.22; 0.95-1.56)	No differences in the OHSS rates; total rec-hFSH dose, estradiol levels, or number of oocytes retrieved.
	Unselected	Antagonist	3	216	No differences in CPR <sup>1</sup> (0.79; 0.26-2.43) or OPR <sup>1</sup> (0.83; 0.39-1.8)	
	Poor Responders	Agonist	3	310	Higher OPR <sup>1</sup> with LH supplementation (1.85; 1.1-3.11)	
Oliveira et al., 2007	Unselected	Agonist	4	1,227	No difference in CPR <sup>2</sup> (1.1; 0.85 - 1.42)	Fewer days of stimulation, lower total rec-hFSH dose, and higher estradiol levels on the hCG administration day in pts. receiving rec-hLH. No difference in the number of oocytes, IR and miscarriage rates.
Baruffi et al., 2007	Unselected	Antagonist	5	434	No difference in CPR <sup>2</sup> (0.89; 0.57-1.39)	No difference in the total rec-hFSH dose, stimulation duration, number of oocytes, IR, or miscarriage rates. Higher estradiol levels and greater number of mature oocytes in pts. receiving rec-hLH.
Kollianiakis et al., 2007	Unselected	Agonist; Antagonist	7	701	No differences in LBR <sup>1</sup> (0.92; 0.65-1.31) or CPR <sup>1</sup> (0.86; 0.61-1.20)	No difference in total rec-hFSH dose, stimulation duration, number of oocytes, or fertilization rates.
Hill et al., 2012	≥35 yo.	Agonist; Antagonist	7	603	Higher IR (OR = 1.36; 95% CI: 1.05 to 1.78, I <sup>2</sup> = 12%) and CPR <sup>1</sup> (1.37; 1.03-1.83; I <sup>2</sup> = 28%) with LH supplementation	No difference in total rec-hFSH dose, stimulation duration, number of oocytes, or fertilization rates.
Bosdou et al., 2012	Poor Responders	Agonist; Antagonist	7	902	No difference in CPR <sup>1</sup> : (RD = 6%; 95% CI: -0.3; +13%); Higher LBR <sup>1</sup> (RD = +19%; 95% CI: +1; +36%) with LH supplementation	No difference in the total rec-hFSH dose, stimulation duration, or number of oocytes.
Fan et al., 2013	Poor Responders	Agonist	3	458	No difference in OPR (1.30; 0.80-2.11)	No difference in the total rec-hFSH dose, stimulation duration, number of oocytes, or cycle cancellation.

CPR: clinical pregnancy rate; OPR: ongoing pregnancy rate; LBR: live birth rate; IR: implantation rate.

OR: odds ratio; CI: confidence interval; RD: risk difference.

OHSS: ovarian hyperstimulation syndrome.

rec-hFSH: recombinant human follicle-stimulation hormone; rec-hLH: recombinant human luteinizing hormone.  
<sup>1</sup>per randomized woman; <sup>2</sup>per oocyte retrieval.



when recombinant LH was added to the stimulation regimen (55). Along the same lines, Mochtar et al., while specifically studying poor responders, demonstrated the benefit of adding rec-hLH during COS. These authors pooled three RCTs, which included 310 participants, and showed higher ongoing pregnancy rates (OR = 1.85; 95% CI: 1.1 to 3.11) in patients treated with a combination of rec-hFSH and rec-hLH compared with rec-hFSH alone (91). In contrast, Fan et al. also studied poor responders by pooling three RCTs and found no differences in ongoing pregnancy rates with LH supplementation (OR = 1.30; 95% CI: 0.80 to 2.11). Furthermore, a significant difference was not detected for the number of oocytes retrieved, the total rec-hFSH dose, the total stimulation duration and the cycle cancellation rate between the study and control groups (113). Finally, in a meta-analysis that included 7 RCTs and 603 patients, Bosdou et al. showed conflicting results following LH supplementation. Although the results were not statistically significant in their study, the magnitude of the size effect and width of the 95% CI for clinical pregnancy (RD = +6%; 95% CI: -0.3 to +13%;  $p = 0.06$ ) suggested a potential clinical benefit of LH supplementation. Despite the heterogeneity of the studies included with regard to patient selection, stimulation protocol, and dose of rec-hLH, the results from a single RCT revealed significantly higher live birth rates after IVF upon LH supplementation (RD = +19%; CI: +1 to +36%) (54).

In summary, existing evidence suggests that LH supplementation could benefit select patient subgroups, but the results should still be interpreted with caution for several reasons. First, the definition of a poor ovarian response was not uniform among studies. Second, the ovarian stimulation protocols differed in dosing, the LH supplementation onset, and the duration of stimulation. Third, the number of completed trials remains low. Finally, many questions have not been fully answered, including how to identify patients who would benefit from LH supplementation, how much LH is necessary, when to begin LH supplementation, and which type of LH activity is best, i.e., recombinant LH or hCG derived (54,99,113).

Notably, an open-label RCT compared the clinical efficacy of LH supplementation using either recombinant LH (a combination of follitropin alfa + lutropin alfa at a fixed ratio of 2:1) or hCG-driven LH activity (HP-hMG) in a small group of women with pituitary insufficiency. Although the proportion of patients who reached ovulation did not differ between the groups (70% vs. 88%, respectively), the pregnancy rate was significantly higher in the rec-hLH group (55.6% vs. 23.3%;  $p = 0.01$ ) (114). Similarly, in an IVF RCT involving 106 women with a normal ovarian reserve and low endogenous LH levels ( $<1.2$  IU/L), a shorter stimulation duration ( $10.9 \pm 1.1$  vs.  $14.1 \pm 1.6$  days;  $p = 0.013$ ) and more retrieved oocytes ( $7.8 \pm 1.1$  vs.  $4.1 \pm 1.2$ ;  $p = 0.002$ ) were observed in patients who received follitropin alfa + lutropin alfa 2:1 compared with HMG. At the end of stimulation, the estradiol level ( $1,987 \pm 699$  pg/mL vs.  $2,056 \pm 560$  pg/mL), pregnancy rate per cycle (28.3% vs. 29.3%) and implantation rate (12.1% vs. 12.2%) did not differ between the groups. However, a higher cancellation rate due to an excessive response was observed in women receiving follitropin + lutropin alfa (11.1% vs. 1.7%;  $p = 0.042$ ) (115). Finally, a large matched case-control study involving 4,719 IVF patients showed that the probability of a clinical pregnancy was higher in patients who used the

fixed combination of rec-hFSH and rec-hLH at a 2:1 ratio (32%) when compared with patients who used hMG (26%;  $p = 0.02$ ) (116). Not surprisingly, these limited data suggest that the fixed rec-hFSH plus rec-hLH combination is superior to hMG. Unlike rec-hLH, LH activity in hMG is derived from hCG, which has a markedly longer half-life and greater binding affinity for LH/hCG receptors compared with LH (57). Lower expression of the LH/hCG receptor gene as well as the genes involved in cholesterol and steroid biosynthesis has been observed in GCs from patients treated with hMG when compared with FSH-treated patients (58). Constant ligand exposure to hCG during the follicular phase likely produces these effects. In fact, LH receptor down-regulation for up to 48 h has been reported in animal models after hCG administration (59), but the clinical implications of these findings have not been fully elucidated in humans (61).

## ■ FUTURE PERSPECTIVES

Low molecular weight (LMW) gonadotropins are currently under investigation. These non-peptide molecules have *in vivo* bioactivity when administered orally (117,118). The first LMW peptides with bioactivity for FSH receptors were described in 2002. In recent years, other compounds have been identified, including biaryl diketopiperazines, thienopyrimidines, dihydropyridines, and thiazolidinones. In the meantime, peptides with agonist activity for LH have also been identified (117,118). However, the clinical efficacy of these compounds has not been determined. FSH and LH receptors compose a subgroup of G protein-coupled receptors with seven transmembrane domains and a large N-terminal extracellular region, which is the predominant site for hormone binding (117,119,120). Receptor activation requires that the hormone binds the N-terminal region, thus leading to intramolecular signal transduction from the ligand-receptor complex to the transmembrane domains. Current LMW gonadotropins are allosteric compounds that presumably interact with the transmembrane domains instead of the N-terminal region. As such, the signaling pathways induced differ from those induced by the native, orthosteric ligands. Recently, a newly developed LMW agonist for the FSH (and LH) receptor has been shown to be orally bioactive in animal studies (118,119). In the future, gonadotropins could be taken orally and replace the injectable forms currently available (117,118).

## ■ REVIEW CRITERIA

An extensive search of studies examining the use of gonadotropins in assisted reproduction was performed using ScienceDirect, Google Scholar, PubMed, and MEDLINE. The overall strategy for identification and data extraction in the study was based on the following key words: "gonadotropins", "follicle-stimulating hormone", "luteinizing hormone", "human chorionic gonadotropin", "controlled ovarian stimulation", and "assisted reproductive technology". Only articles published in English were considered. The end date for the searches was May 2013. Data that were solely published in conferences or meeting proceedings, websites, or books were not included. Websites and book-chapter citations provided conceptual content only. Concerning the clinical efficacy of gonadotropins, only meta-analytic studies published after 2005 were included.



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## AUTHOR CONTRIBUTIONS

Both authors were involved in the data collection, critical analyses for factual and scientific content, and the drafting and revision of the manuscript.

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