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Effect of Recombinant Human FSH on Ovulation, Pregnancy and *In Vitro* Fertilization in Androgen-Sterilized Mice

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The effect of a new rhFSH, PG-0801, on oocyte quality, ovulation and in vitro fertilization (IVF) was examined in androgen-sterilized mice. Experimental sterility was induced by a single subcutaneous injection of testosterone propionate (TP, 1 mg/head) into 5 day old female mice. Ovulation was generated in the 10 to 13-week old TP-injected mice by a subcutaneous rhFSH injection (1, 5 or 10 IU/head) followed 48 hours later by a second rhFSH injection (1, 5 or 10 IU/head). For comparison, a subcutaneous PMSG (5 IU/head) injection was used for folliculogenesis and a hCG (5 IU/head) injection was used for ovulation. These were administered using the same protocol. The eggs were harvested from the oviducts and counted 17 to 20 hours after the second injection. IVF was performed by adding sperms (2×10^5/ml to 2×10^6/ml) to determine the functional activity of the eggs, and the fertilization rate was measured. In addition, the pregnancy rate and fetal development were examined after 15-17 days of gestation. The number of oocytes recovered from the rhFSH/rhFSH group increased dose-dependently and was slightly higher than that of the PMSG/hCG group. The pregnancy rates of the group receiving 1, 5, and 10 IU of rhFSH/rhFSH were 50%, 66.7%, and 75%, respectively, which were significantly higher than that of the control (untreated) group (0%). The numbers of viable fetuses in the 1, 5, and 10 IU/head of the rhFSH/rhFSH group (8.0 ± 1.50, 8.9 ± 1.02, and 8.9 ± 1.12 fetuses/dam, respectively) were comparable to that of the 5 IU/head PMSG/hCG group (9.4 ± 0.94). The mice receiving rhFSH/rhFSH and PMSG/hCG showed similar fertilization rates (around 65%) via the IVF procedure. These results demonstrate that a new rhFSH, PG-0801, may be useful for inducing ovulation in functionally infertile patients and for superovulation in ovulatory patients participating in assisted reproductive technology (ART) programs.

Key words: Recombinant human FSH (rhFSH), PG-0801, Androgen-sterilized mice, Ovulatory induction, *In Vitro* fertilization (IVF)

INTRODUCTION

It is becoming increasingly clear that FSH has a distinct, but complimentary, roles in the normal process of follicular growth, and oocyte development. The ovulatory activity of FSH has been researched since the mid-1960's when Lostroh and Johnson (1966) used ovine FSH to induce ovulation in intact and hypophysectomized rodents (Lostroh *et al.*, 1966). Other studies (Armstrong *et al.*, 1988; Filicori *et al.*, 1990; Stern *et al.*, 1970) over the last 20 years confirmed the ability of FSH to induce ovulation using either ovine or porcine FSH. It has been reported that FSH itself induces a temporal increase in the lutenizing hormone (LH) immediately before ovulation (McClintock *et al.*, 1968; Parkening *et al.*, 1982; Hoff *et al.*, 1983), which is more effective in inducing oocyte maturation in cultured follicles than LH being applied directly (Neal *et al.*, 1975). In addition, FSH plays an important role in preovulatory events, such as the production of the plasminogen activator by granulosa cells. This then converts the plasminogen to plasmin, which weakens the follicle wall (Strickland *et al.*, 1976). Moreover, FSH, but not LH, is known to stimulate cumulus expansion through the synthesis and deposition of a hyaluronic acid matrix, thereby allowing the oocyte-cumulus cell mass to become free floating in the antral fluid prior to follicular rupture (Epping *et al.*, 1979). Based

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on these physiological roles of FSH, recombinant human FSH (rhFSH) is widely used for inducing ovulation and pregnancy in anovulatory infertile patients and for developing multiple follicles in ovulatory patients participating in assisted reproductive technology (ART) programs.

Recently, ProGen (Kyunggi, Korea) established a stable cell line producing rhFSH by transfecting Chinese hamster ovary (CHO) cells with a plasmid containing the two subunit genes of human FSH. A new rhFSH, PG-0801, produced by this recombinant technology has a high biochemical purity (≥99%) and a high specific biological activity (~13,800 IU/mg). Further characterization of the cell line has been performed and it has been confirmed that the amino acid composition and sequence of PG-0801 matches those of natural human FSH. In this efficacy study, the effect of PG-0801 on ovulation and subsequent pregnancy was investigated in experimentally sterilized mice.

MATERIALS AND METHODS

Test materials

Recombinant human FSH (rhFSH, code name : PG-0801, lot. FPC-10-05) was obtained from ProGen. The biochemical purity of the material according to electrophoresis was 99.5%. PMSG, hyaluronidase and hCG were obtained from the Sigma Chemical Company (St. Louis, MO., USA). Testosterone propionate (TP, Testo™) was obtained from Samil Pharmaceutical Co. Ltd. (Kyunggi, Korea). All other chemicals were of analytical grade or of the highest quality commercially available.

Animals

Six-week-old ICR female and male mice (Charles River Japan Inc., Tokyo, Japan) were grouped and housed together in a temperature-controlled animal care room with a 12 hour light/dark cycle. The mice were given free access to water and laboratory chow. After mating, the pregnant animals were monitored for the day of delivery. The pups were counted and sexed, and all males were removed from the litters at 5-day old. One mg of testosterone propionate (TP) was injected subcutaneously into the 5-day-old female pups to cause androgen-induced sterility. All pups were weaned from their mother at 21-days-old and kept under the same controlled conditions until needed.

Induction of ovulation

One hundred and twenty androgen-sterilized mice (10-13 weeks old) were divided into 3 groups. Ovulation was induced either by a PMSG/hCG or a rhFSH/rhFSH injection. Briefly, the sterilized mice were selected at random stages of their estrus cycle and injected S.C. with 5 IU of PMSG/head in 0.2 ml saline at 16:00 h. After 48 hours, the animals were injected S.C. with 5 IU of hCG/head. In the rhFSH/rhFSH group, the sterilized mice were injected S.C. with 1 IU, 5 IU, or 10 IU of rhFSH, and the animals received the same rhFSH dose S.C 48 hours later. The control mice received 0.2ml saline S.C.

Mating and fetal assessment

Mating was allowed in order to examine the reproductive function and the fetuses by housing ovulation induced ICR female mice (n=60) together with the ICR males immediately after the second injection. Day 0 of pregnancy was determined as the day a vaginal plug was observed. The coupled female mice were sacrificed on days 15-17 of pregnancy and the uterus was examined for the presence of fetuses. The fetuses removed from their placental and membrane attachments were examined grossly for viability and development.

Oocyte collection

Approximately 18 hours after the second injection for ovulation, the animals were randomly selected from each group (12 mice/group) and sacrificed by a cervical dislocation. The oviducts and ovaries were removed and placed in human tubal fluid (HTF) medium supplemented with 7.5 mg/ml of bovine serum albumin (BSA, Fraction V, No. A-4503: Sigma Chemical Co., St. Louis, MO., USA) and antibiotics (penicillin 100 U/ml, streptomycin sulfate 50 μg/ml). The medium was stored in an incubator (humidified, 5% CO_2, 37°C) 8-10 hours prior to the experiments. The oviducts and ovaries were trimmed with the aid of a dissecting microscope, and the ovarian bursae were examined. The oviducts were punctured and flushed to ensure the recovery of all oocytes. After the oocytes had been retrieved, 0.1% hyaluronidase was added to disperse the cumulus cells, and the oocytes were counted.

In vitro fertilization

Sperm was collected from mature male ICR mice by gently squeezing the vas deferens and the cauda epididymis removed from the bilateral testes. The collected sperm was incubated for 1.5 hours for capacitation in a loosely capped round-bottomed tube (No. 2058, Falcon: Beckton Dickinson Labware, Oxnard, CA., USA) with 1.0 ml HTF medium. Sperm motility was then examined

microscopically and the sperm concentration was assessed using a Maklar counting chamber (Sefi-Medical Instruments LTD., Haifa, Israel).

Twelve and a half hours after the second hCG or rhFSH injection, the animals were sacrificed and the oviducts were placed in an organ tissue culture dish (No. 3037, Falcon) containing HTF on a warming plate (37°C). The swollen ampulae were lanced with a sterile 21 G needle, which allowed the cumulus enclosing the oocytes to be released directly into the medium. A number of eggs collected from each mouse were transferred to the well of a tissue culture plate (No. 662160, Greiner Labortechnik, Germany) containing 0.5 ml HTF, and incubated until insemination. Thirteen and a half hours after the second injection of hCG or rhFSH, insemination was performed by adding sperms at a concentration of 2×10^5 to 2×10^6 sperms/0.1ml to each well. Incubation was then continued for 10 hours at 37°C in a humidified 5% CO_2 and 95% O_2 atmosphere. The eggs were washed twice in the medium, counted and monitored hourly using bright-field microscopy. Fertilization was confirmed by the presence of at east 2 pro-nuclei in the cytoplasm and a second polar body.

Statistics

The number of oocytes and fetuses per treatment group were compared using one-way ANOVA or an unpaired Students t test, where appropriate. When ANOVA indicated differences between the groups, post-hoc analysis was performed using the Student Newman-Keuls Procedure. The ovulatory and pregnancy rates were assessed by a Chi-Squre test. All results are reported as a means the standard error (SEM).

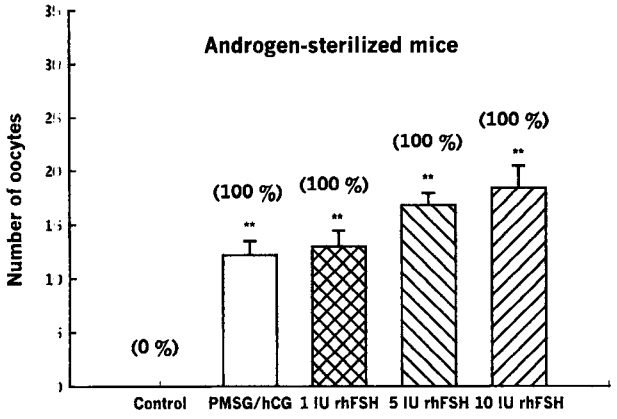

Fig. 1. Number of eggs retrieved from the oviducts after ovulation with PMSG/hCG (5 IU/5 IU) or rhFSH/rhFSH in the androgen-sterilized mice. The ovulation rate, the percentage of ovulated mice out of the total number of mice, is shown in parenthesis. The values represent a mean SEM. **p<0.01 compared to control.

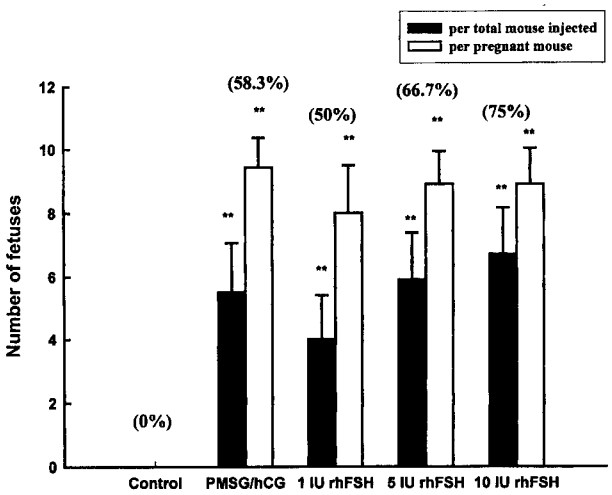

Fig. 2. Reproductive response of the mice stimulated (first injection) and ovulated (second injection) with PMSG/hCG (5 IU/5 IU) or rhFSH/rhFSH in the androgen-sterilized mice. The pregnancy rate, the percentage of pregnant mice out of the total number of mice mated, is given in parentheses. The values represent a mean SEM. **p<0.01 compared to control.

RESULTS

Effect of rhFSH on ovulation in sterilized mice

The mice receiving rhFSH/rhFSH or PMSG/hCG, ovulated 100% of the time, while none of the control mice injected with saline ovulated (Fig. 1). The number of oocytes recovered from the mice receiving 1, 5, and 10 IU/head of rhFSH was 13.1 ± 1.68, 16.8 ± 1.11, and 18.4 ± 2.15, respectively. In particular, the number of oocytes in the rhFSH/rhFSH groups increased in a dose-dependent manner and there were considerably more oocytes than in the control group (0, $p<0.01$). Similarly, the number of oocytes in the PMSG/hCG group (12.2 ± 1.38) was significantly higher than in the control group ($p<0.01$).

Effect of rhFSH on pregnancy in sterilized mice

The effect of rhFSH on the reproductive response was determined by examining the extent of fetal development.

Table 1. Number and fertilization rate for eggs retrieved from the oviducts after ovulation with PMSG/hCG or rhFSH/rhFSH.

Group	Dose	Number of mice	Fertilization rate (%)
Control	0 IU/0 IU	12	0/0*(0 ± 0%)#
PMSG/hCG	5IU/5IU	12	97/150*(65 ± 4%)#
RhFSH/rhFSH	1IU/1IU	12	106/161*(67 ± 8%)#
RhFSH/rhFSH	5IU/5IU	12	118/173*(68 ± 5%)#
RhFSH/rhFSH	10IU/10IU	12	117/180*(66 ± 7%)#

No. of fertilized oocytes to No. of collected oocytes
#Mean ± SEM

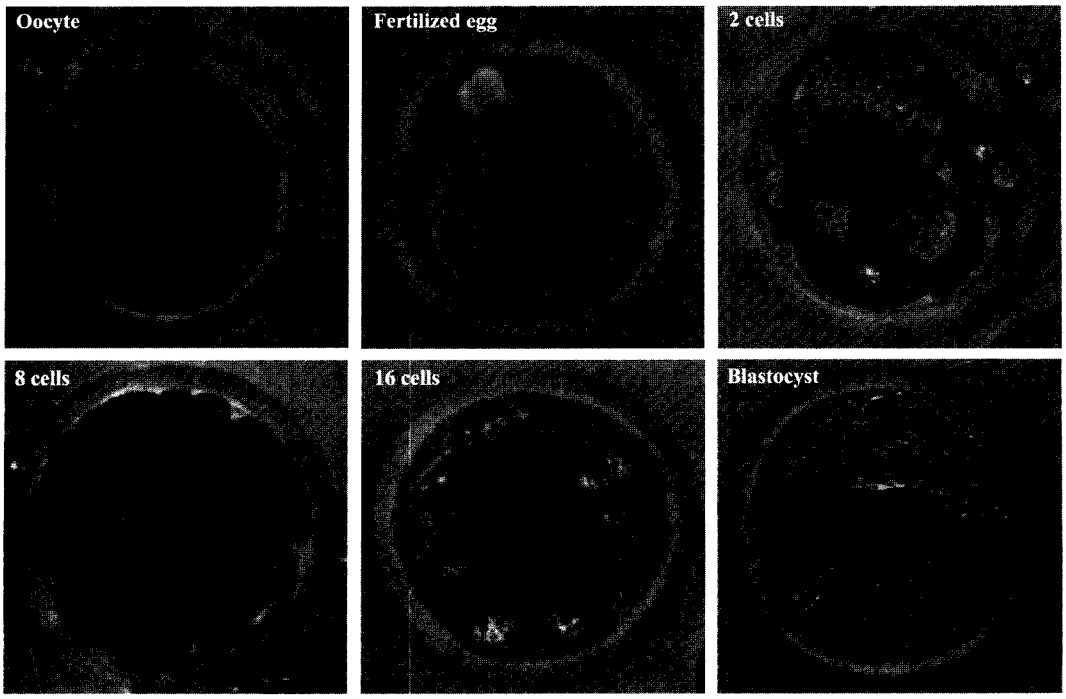

Fig. 3. The early stage of embryonic development in vitro. The zygotes were obtained after *in vitro* fertilization of the recovered oocytes. (Original magnification, x 100.)

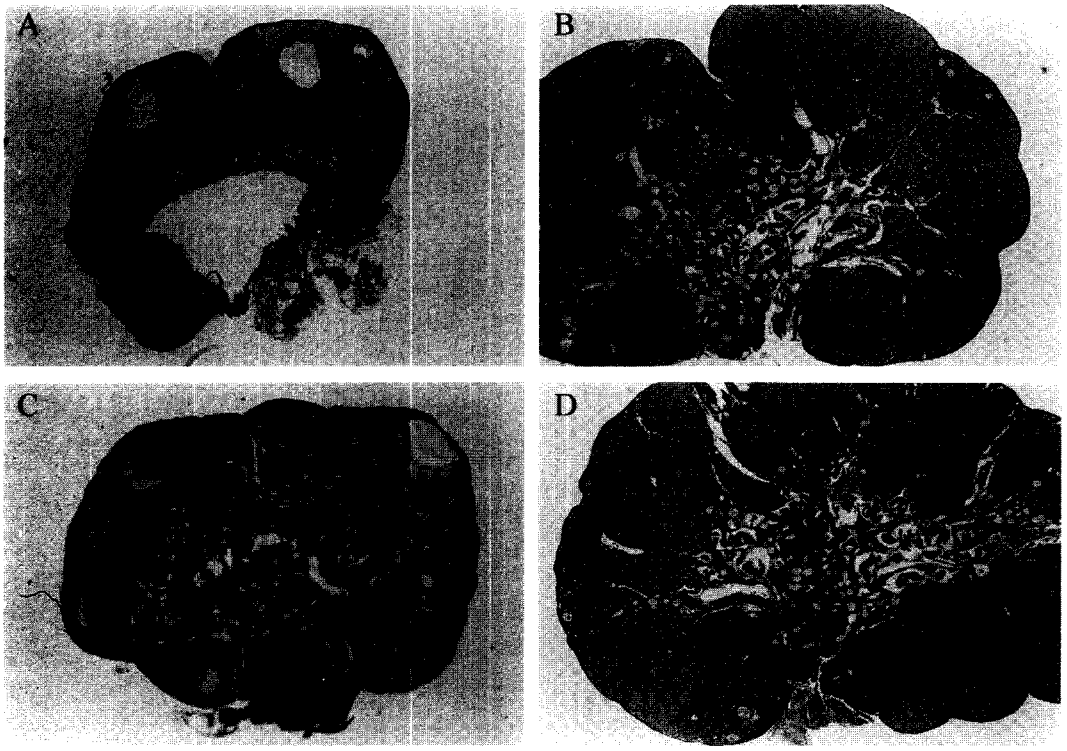

Fig. 4. A) Control ovary. No corpora lutea (CL) and a few antral follicles (AF) can be seen. Two cystic follicles (CF) are particularly prominent. B) PMSG/hCG treatment. The ovary is visibly larger than the control (*Fig.* A), and many corpora lutea are apparent. C) rhFSH/rhFSH (1 IU/1 IU) treatment. The ovary is also visibly larger than the control, and numerous antral follicles appear throughout. D). rhFSH/rhFSH (10 IU/10 IU) treatment. The ovary is visibly larger than the control, and is occupied by large corpora lutea and antral follicles. (X 40 magnification for all of the above).

Each androgen-sterilized female was mated with a fertile male after inducing ovulatory with 1, 5 and 10 IU of rhFSH/rhFSH or 5 IU PMSG/hCG. The mice receiving the 1, 5 and 10 IU of rhFSH/rhFSH exhibited pregnancy rates of 50%, 66.7% and 75% and 8.0 ± 1.50, 8.9 ± 1.02, and 8.9 ± 1.12 fetuses/dam, respectively (Fig. 2). The PMSG/hCG-injected mice exhibited a pregnancy rate of 58.3% with 9.4 ± 0.94 fetuses/dam. In contrast, none of the control group was pregnant. All fetuses removed from the rhFSH/rhFSH group and PMSG/hCG group were alive and appeared to be normal.

In vitro fertilization

The fertilization rate in each group is shown in Table 1. The fertilization rate of the eggs recovered from the PMSG/hCG group (65%) was comparable to those of the 1, 5, and 10 IU rhFSH/rhFSH groups (67%, 68%, and 66% respectively). The fertilized eggs showed polar bodies and zygote cleavage (Fig. 3).

Effect on ovarian histology

A histological examination of the control mice revealed single or multiple follicular cysts in their ovaries (Fig. 4). The cysts were lined with one to four layers of cuboidal granulosa cells, which were rested upon a smooth basement membrane. Occasionally, the cysts were lined by a single layer of flattened epithelial cells, which was surrounded by a thin capsule of fibrous connective tissue. The cysts contained pale eosinophilic proteinaceous material or blood, a portion containing degenerated oocytes, and large foamy vacuolated or hemosiderin-laden macrophages. In the control group, two follicular cysts with a few primordial or secondary follicles were observed in the ovaries. In contrast, the PMSG/hCG- and rhFSH/rhFSH-injected groups contained many antral follicles and corpus lutea but relatively few cystic follicles. In addition, the ovaries were visibly larger than those of the control group (Fig. 4).

DISCUSSION

There have been many studies on superovulation performed on mammals. Ovulation in mammals is preceded by surges in the two pituitary gonadotropins, LH and FSH. However, the role LH plays in folliculogenesis is unclear. Many researchers have demonstrated that rhFSH alone stimulates antral and preantral follicle growth in hypophysectomized rats (Takanori *et al.*, 1994), cynomolgus monkeys treated with a GnRH antagonist (Karnitis *et al.*, 1994), and hypogonadotropic women (Schoot *et al.*, 1992). However, E_2 and androstenedione production were significantly low. Recently, Schats *et al.* (2000) demonstrated that rhFSH is more effective than highly purified human urinary FSH at inducing multifollicular development. Montgomery *et al.* (1993) reported that pure FSH alone induced ovulation and the oocytes produced were capable of fetal development in the absence of any exogenous LH. Therefore, in this study, a novel rhFSH, PG-0801, was examined to determine if it could induce both ovulation and pregnancy in androgen-sterilized mice.

The number of oocytes retrieved from the rhFSH/rhFSH group increased with increasing dose compared to that of control group. PMSG/hCG treatment increased the number of oocytes retrieved from each oviduct. These results indicate that rhFSH/rhFSH treatment can induce ovulation in the androgen-sterilized mice used as the model for anovulation. IVF was then performed to determine the functional activity of the eggs retrieved from both the rhFSH/rhFSH group and the PMSG/hCG group. The fertilization rates of the oocytes following IVF were similar for the both groups (near 65%). Xiaowei *et al.* (2000) reported that FSH is essential for follicular growth (FSH threshold) in the small preantral follicles, but there was no statistically difference between the normal and androgen-sterilized mice.

In addition, rhFSH/rhFSH treatment appeared to increase both the pregnancy rates and the number of fetuses in this study. The pregnancy rates for the group receiving 5 and 10 IU of rhFSH/rhFSH were higher than that for the group receiving 5 IU of PMSG/hCG. Montgomery *et al.* (1988) also reported that PMSG/rFSH-ovulated oocytes have the ability to undergo cleavage to the blastocyst stage and the oocytes were capable of developing into a fetus. Recently, the treatment of polycystic ovarian syndrome (PCOS) has progressed with the advent of new therapeutic modalities. However, there are still substantial difficulties in managing of this syndrome. One of the clinical problems associated with PCOS is the high incidence of ovarian hyperstimulation syndrome (OHSS) (Schenker et al., 1978) during ovulatory induction by gonadotropin therapy. To reduce these serious risks, more elaborate methods, such as the use of purified FSH (Seibel et al., 1985), careful monitoring of follicular growth (Shoham et al., 1991), rapid sera hormone assays (Shoham et al., 1991), and a new regimen based upon pharmacokinetics for drug administration should be adopted (Mizunuma et al., 1991). These techniques have been used clinically, but with conflicting results (Larsen et al., 1990; Homburg et al., 1990; Sagle et al., 1991). Therefore, the availability of highly purified rhFSH has increased the treatment of anovulation in women with PCOS.

It has been documented that FSH stimulates folliculogenesis in both animals and humans (Seibel *et al.*, 1985; Sagle *et al.*, 1991; Nuti *et al.*, 1974). Montgomery *et*

al. (1993) suggested that FSH alone without exogenous LH might also initiate events leading to follicular rupture (ovulation induction) and meiosis resumption in mice. Galway *et al.* (1990) using recombinent FSH (rFSH), reported that both TPA and its messenger RNA (mRNA) reached peak levels 12 hours a after rFSH injection. However, although it is clear that LH usually triggers ovulation, it has been suggested that the FSH surge may act synergistically with LH (Hoff *et al.*, 1983; Schwartz *et al.*, 1974). The role of the primary FSH surge has been linked to preovulatory events such as tissue plasminogen activator (TPA) production and cumulus expansion through the synthesis and deposition of a hyaluronic acid matrix (Strickland *et al.*, 1976). Furthermore, the addition of recombinant LH to recombinant FSH in pituitary-suppressed women undergoing ART does not improve the ovarian response, and may even have a negative impact on oocyte maturation and fertilization (Balasch *et al.*, 2001). However, the mechanism of ovulation induction by rhFSH remains unclear.

In conclusion, the new rhFSH, PG-0801, can has an effect on ovulatory induction and the high pregnancy rate in androgen-sterilized mice. However, because the pathophysiology of the androgen-sterilized mice used as an anovulation model is not identical to that of PCOS in humans, additional studies to determine whether or not a rhFSH/rhFSH regimen can normalize the ovulatory function, fetal development and fertilization in humans are recommended.

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