Comparative Medicine
Copyright 2023
by the American Association for Laboratory Animal Science

Vol 73, No 2
April 2023
Pages 145–152

Original Research

Bioactivity and Pharmacodynamics of X002, a Follicle-stimulating Hormone–IgG4 Fc Fusion Protein

Guili Xu,[1,*] Yi Yang,[1] Yunhui Liu,[1] Fang Chen,[2] Lihou Dong,[2] Deyou Wan,[1] Hongjie Li,[1] Cuima Yang,[1] and Xin Gao[1,*]

Current follicle-stimulating hormone (FSH) drugs meet safety criteria but have suboptimal efficacy, poor patient compliance, and high cost. Alternative FSH-like drugs would help to meet the high market demand. Here, we evaluated X002, an FSH–Fc fusion protein, for bioactivity and half-life in vitro and in vivo. In all cases, the effects of X002 were compared with those of a commercially available short-acting FSH recombinant hormone. First, female Kunming mice (age, 21 to 24 d) were stimulated with pregnant mare serum gonadotropin (PMSG) for 46 h, after which naked oocytes were harvested, treated with X002 or the comparison agent at 37 °C for 4 h, and then evaluated for germinal vesicle breakdown. Second, cumulus–oocyte complexes (COC) were collected from PMSG-stimulated mice and cocultured with X002 or the comparison agent for 14 h; the COC diameters were then measured, and the expression of genes involved in COC expansion were evaluated using quantitative RT-PCR analysis. Third, to assess the pharmacokinetics of X002, female Sprague–Dawley rats (age, 6 to 8 wk) were injected subcutaneously with X002 or the comparison agent; serum samples then were collected at various times and assessed via ELISA. Fourth, to evaluate X002 pharmacodynamics, 26-d-old female Sprague–Dawley rats were treated with X002 or the comparison agent; 84 h later, the rats were stimulated with human chorionic gonadotropin (hCG). At 12 h after hCG injection, euthanasia was performed. Ovaries were removed and weighed, and serum levels of estradiol and progesterone were measured. Finally, to assess superovulation, the oocytes in the fallopian tubes were counted at 108 h after in vivo treatment of rats with X002 or the comparison agent. The data show that X002, a long-acting agent, promoted germinal vesicle breakdown and COC expansion in vitro and in vivo ovarian weight gain and superovulation to a degree similar to the short-acting comparison agent.

Abbreviations: COC, cumulus-oocyte complex; FSH, follicle-stimulating hormone; GVBD, germinal vesicle breakdown; hCG, human chorionic gonadotropin; PMSG, pregnant mare serum gonadotropin

DOI: 10.30802/AALAS-CM-22-000001

Follicle-stimulating hormone (FSH) is a glycoprotein hormone that consists of an α subunit and a β subunit and is secreted by the gonadotropic cells of the anterior pituitary gland.[9] The main physiologic functions of FSH are to promote the development and maturation of ovarian follicles in females and to regulate Sertoli cell function in males. FSH products, especially recombinant FSH in gonadotropin-releasing hormone agonists or antagonists, are used for clinically assisted human reproduction.[4] When combined with ovulation-induction agent such as human chorionic gonadotropin (hCG), FSH facilitates the production of mature oocytes.[6] Currently, women seeking assisted reproduction are injected daily with short-acting FSH-related drugs or receive a single injection of a long-acting FSH agent.[10] The FSH-related products currently available are urine-derived FSH or recombinant hormones (for example, short-acting Gonal-f [Merck, Darmstadt, Germany] and Puregon [Organon, Oss, Netherlands] or long-acting Elonva [Merck]). All of these FSH products are safe, function similarly to the FSH produced by the body, and are clinically useful for assisted reproductive technology, but they can have suboptimal efficacy (potency and half-life), poor patient compliance, and high cost.

To overcome the limitations of currently available FSH drugs, alternatives have been designed that fuse one or both FSH subunits to the Fc component (single chain or heterodimer) of human IgG1,[8] a carboxy-terminal peptide of hCG,[7] or the Fc of human IgG4.[5] However, these products not available in amounts sufficient to meet the current market demand. Therefore, to aid assisted reproduction, we used genetic engineering technology to fuse the Fc segment of human IgG4 with FSH (compound X002; molecular weight, 87.3 kDa; purity, ≥ 99%; Figure 1). We hypothesized that our novel FSH-related product X002 would have favorable bioactivity, a prolonged half-time, and few side effects. In the current study, we tested the bioactivity and pharmacodynamics of X002 in vitro and in vivo.

Materials and Methods

Drugs and reagents. X002 was manufactured by United Power Bioengineering and Biotechnology (production batch no. X0020220171205; Beijing, China) and developed by Beijing Qikang Xingye Biopharma Technology (Beijing, China). In all

Submitted: 05 Jan 2022. Revision requested: 14 Mar 2022. Accepted: 31 Mar 2022.
[1]Beijing Qikang Xingye Biopharma Technology, Beijing, China, and [2]United Power Pharma Tech, Beijing, China

**Corresponding authors. Guili-Xu (Email: 296881485@163.com) and Xin-Gao (Email: gaox_amms@126.com)*

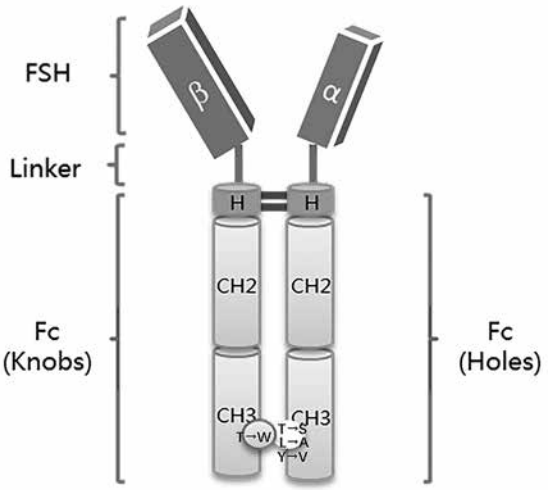

Figure 1. Schematic of the X002 fusion protein. The α and β subunits of FSH are fused to the N terminus of the Fc fragment of human IgG4 (CH2 and CH3 domains, respectively).

studies described below, the effects of X002 were compared with those of the clinically used short-acting recombinant hormone Gonal-f (hereafter called the comparison agent; Merck Serono, Darmstadt, Germany)

Pregnant mare serum gonadotropin (PMSG) was purchased from Beijing Solarbio Science and Technology (product no. P9970; Beijing, China), and hCG was purchased from ProSpec–Tany TechnoGene (product no. HOR-250; Rehovot, Israel).

Equipment and instruments. Equipment and instruments used in this study included a Cobas4000 automatic analyzer (Roche Diagnostics, Shanghai, China), stereo microscope (model no. SMZ800N; Nikon Imaging Instrument Sales, Beijing, China), a scale for weighing rats and samples (model no. YP2002; Shanghai Yueping Scientific Instrument [Suzhou] Manufacturing, Jiangsu, China), and a real-time fluorescence-based quantitative PCR system (model no. ABI 7500Fast; Thermo Fisher Scientific, Shanghai, China).

Experimental animals. The animals used in this study included SPF female Sprague–Dawley rats (ages 26 d and 6 to 8 wk) and a total of 120 SPF female Kunming mice (age 21 to 24 d) purchased from Beijing HFK Bioscience (Experimental Animal Production License no. SCXK [Beijing] 2014-0004; Beijing, China). The animals were housed in IVC systems under a constant temperature of 20 to 26 °C, constant humidity of 40% to 70%, and an alternating 12:12-h light:dark cycle. They had free access to a standard maintenance diet and sterilized drinking water. All animal experiments conformed to the *AVMA Guidelines for the Euthanasia of Animals*[1] and the China Laboratory Animal Guidelines for Euthanasia (no. GB/T 39760-2021), were approved by the Committee of Animal Experiments and Experimental Animal Welfare of Beijing Qikang Xingye Biopharma Technology (approval number: IACUC2018-0002) and were designed and conducted in strict accordance with 3R principles of reduction, replacement, and refinement.

Detection of germinal vesicle breakdown (GVBD) in mouse oocytes. Female Kunming mice (age, 21 to 24 d) were injected subcutaneously with PMSG (5 IU/animal); 46 h later, the mice were euthanized with CO_2 (30% volume per minute displacement rate of 100% CO2 in an induction chamber) and the ovaries were harvested. A pair of 30-gauge needles attached to disposable syringes were used to puncture the antral follicles under a stereomicroscope to retrieve cumulus–oocyte complexes (COC) from the ovaries and collect naked oocytes.[2] Oocytes (80 to 100 per group) were added to culture medium containing either the short-acting comparison agent or X002 at concentrations of 0.3, 3.0, 30.0, or 300.0 nmol/L[15] and incubated for 4 h at 37 °C. The percentages of germinal vesicle breakdown (GVBD) of the oocytes were then compared between groups treated at the same concentrations.[8] The GVBD percentage was calculated using the following equation:

$$\text{GVBD rate (\%)} = \frac{\text{no. of oocytes in GVBD and 1}^{\text{st}}\text{ polar body phase}}{\text{total no. of oocytes - dying or degenerated oocytes}} \times 100\%$$

Analysis of mouse COC expansion and oocyte gene expression. COCs (n = 80 to 100 per group) were then incubated in culture medium containing the comparison agent or X002 at concentrations of 0.3, 3.0, 30.0, or 300.0 nmol/L[15] for 14 h at 37 °C. After this time, the diameters of COC were compared between the groups treated at the same concentration. Specifically, COC were photographed before and after treatment, and COC diameters were calculated by using Image J software.

Finally, COC in each group were pooled, and total RNA was extracted from each group by using RNA Simple Total RNA Kit (catalog no. DP419, TIANGEN Biotech, Beijing, China) according to the manufacturer instructions. RNA was reverse-transcribed and cDNA synthesized by using Revertaid First-strand cDNA Synthesis Kit (catalog no. K1622, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The cDNA templates from different COC groups were used with AceQ qPCR SYBR Green Master Mix (catalog no. Q121-02, Vazyme Biotech, Jiangsu, China) and appropriate primers (Table 1) in RT-qPCR assays to measure the expression of 7 key genes in the oocyte maturation process (amphiregulin, epiregulin, betacellulin, pentraxin 3, hyaluronan synthase 2, prostaglandin-endoperoxide synthase 2, tumor necrosis factor-inducible gene 6).[15] The individual gene expression levels were determined and normalized to that of β-actin as an internal reference gene. The relative expression level of each gene in an untreated control group was set to 1. The $2^{(-\Delta\Delta Ct)}$ method[15] was used to calculate the relative expression level (fold change) of the corresponding gene in the experimental group.

Pharmacokinetic tests. To evaluate the pharmacokinetics of X002, female Sprague–Dawley rats (n = 8 per group) were injected subcutaneously with X002 at 5, 15, or 45 µg/kg. At 4, 8, 24, 48, and 72 h and at 5, 7, 10, 14, 21, and 28 d after X002 administration, rats were anesthetized with isoflurane (3%). Peripheral blood (100 µL) was then harvested from the orbital venous plexus, allowed to clot, centrifuged at 2000 × g for 20 min at 4 °C, and the serum collected. Serum samples were analyzed for concentration of X002 using an FSH Enzyme Immunoassay Kit (minimum sensitivity, 2.5 mIU/mL; catalog number: BC-1029, BioCheck, South San Francisco, CA). Noncompartmental analysis (WinNonLin version 6.4, Certara, Princeton, NJ) was used to calculate various standard pharmacokinetic parameters (Kel, t1/2, T_{max}, C_{max}, C0, $AUC_{(0\text{-}t)}$, $AUC_{(0\text{-}inf)}$, $AUC_{(t\text{-}inf)}$ %, Vd, CL, and MRT_{inf}).

Rat ovarian weight. Based on the guidelines of the Pharmacopoeia of the Peoples' Republic of China (2015 edition),[11] we used 26-d-old, female Sprague–Dawley rats to analyze the mechanism of action of X002. Rats (n = 10 per group) were randomly grouped and subcutaneously injected with the short-acting comparison agent (3.0 pmol per injection for a total of 7 injections with 12 h between injections) or with 1.6, 3.0, 6.0, or 12.0 pmol X002 as a single injection.[2] At 84 h after treatment, all rats were injected subcutaneously with 3.75 IU hCG (n = 10 rats per group). Serum estradiol and progesterone levels and the ratio of ovarian weight

Table 1. Primer sequences of the target mouse genes used for RT-qPCR analysis[15]

	Primer sequences (5′ → 3′)	PCR product size (bp)
Amphiregulin	GAG GCT TCG ACA AGA AAA CG TTT ATC TTC ACA CAT CTC TTT ATG TAC AG	210
Epiregulin	GCA TCC CAG GAG AAT CCG AG CTC ACA TCG CAG ACC AGT GT	197
Betacellulin	GTA GCA GTG TCA GCT CCC TG ATG CTT GTA CTG CTT GGG GC	206
Hyaluronan synthase 2	GTT GGA GGT GTT GGA GGA GA ATT CCC AGA GGA CCG CTT AT	155
Prostaglandin-endoperoxide synthase 2	TGT ACA AGC AGT GGC AAA GG CCC CAA AGA TAG CAT CTG GA	230
Tumor necrosis factor-inducible gene 6	TTC CAT GTC TGT GCT GCT GGA TGG AGC CTG GAT CAT GTT CAA GGT CAA A	328
Pentraxin 3	GTG GGT GGA AAG GAG AAC AA GGC CAA TCT GTA GGA GTC CA	190
β-actin	GCT CTT TTC CAG CCT TCC TT GTA CTT GCG CTC AGG AGG AG	234

to body weight were evaluated as indices of bioactivity. Rats were euthanized with CO_2 (30% volume per min displacement rate of 100% CO_2 in an induction chamber) at 96 h after the first administration of the corresponding treatment. Then venous blood samples were collected and allowed to clot, and ovaries were harvested and weighed. Estradiol and progesterone levels were quantified via ELISA (Estradiol [rat] ELISA kit, catalog no. K3831, BioVision, Milpitas, CA; Rat Progesterone ELISA kit, catalog no. 80558, Crystal Chem, Elk Grove Village, IL).

Detection of superovulation in rats. Female Sprague–Dawley rats (age 26 d; n = 10 per group) were randomly grouped according to body weight, treated with the comparison agent or X002 as described previously,[15] subcutaneously injected with 20 IU hCG at 84 h after the first injection, and euthanized with CO_2 (30% volume per min displacement rate of 100% CO_2 in an induction chamber) at 108 h after the first drug injection. The fallopian tubes were then removed and dissected, and the oocytes isolated and counted.[15]

Statistical analysis. To ensure sufficient statistical power in our data analysis, we performed a power analysis prior to the study, and thereafter used the appropriate number of samples to provide 80% power to detect a difference in a 2-sided test with P less than 0,95. All data are presented as mean ± SEM or mean ± SD, as appropriate. Prism 8.0 (GraphPad Software, San Diego, CA) was used for graph preparation, and SPSS version 22.0 (IBM, Armonk, NJ) was used for 1-way and 2-way ANOVA. Results were considered statistically significant at a P value less than 0.05.

Results

GVBD rate of mouse oocytes. The percentage of oocytes exhibiting GVBD gradually increased as the concentration of the treatment agent increased and were equivalent between X002 and the comparison agent at concentrations of 0.0 to 30.0 nmol/L (Figure 2). However, at 300.0 nmol/L, the GVBD percentage was lower (P < 0.05) for X002 than that of the comparison agent and was closer to that of 30.0 nmol/L X002. These results show that X002 promoted GVBD in mouse oocytes in a manner similar to that of the comparison agent at concentrations of 0.0 to 30.0 nmol/L but showed a saturation effect at higher concentrations.

Diameter of mouse COC. At 14 h after treatment, the COC diameter of the X002 group was significantly (P < 0.01) larger than that of the untreated control group (Figure 3). Figure 4 shows the differences in average COC diameters before and after the expansion. The average COC diameters increased gradually, with increasing concentrations. However, no statistically significant differences were seen in average COC diameters of the X002 and short-acting comparison drugs at the corresponding concentrations.

RT-qPCR was used to measure the expression levels of key genes for the oocyte maturation process (Figure 5). Oocytes treated with either the commercially available FSH-like agent or X002 showed significantly upregulated expression of key genes as compared with the expression in untreated controls. In particular, the expression of amphiregulin, epiregulin, and prostaglandin-endoperoxide synthase 2 was similar between the 2 FSH-like treatment groups, whereas the expression of betacellulin, pentraxin 3, hyaluronan synthase 2, and tumor necrosis factor-inducible gene 6 was greater (P < 0.05) in X002-treated oocytes but was not dose dependent.

Pharmacokinetics of X002. The results of the pharmacokinetics analysis of X002 in rats are shown in Table 2 and Figure 6. The AUC(0-t) was similar to the dose ratio, indicating linear pharmacokinetic characteristics. These data also show that X002 has a much longer half-life (140 to 262 h) than does the

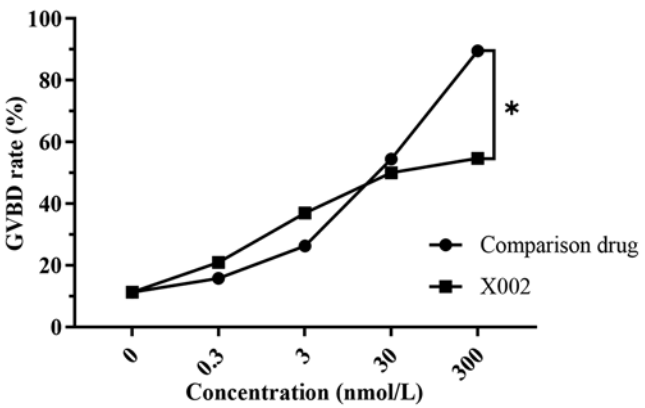

Figure 2. Germinal vesicle breakdown (GVBD) of oocytes treated with X002 or the comparison drug. Female Kunming mice were injected subcutaneously with PMSG (5 IU/animal). Naked oocytes were collected 46 h later and cocultured at 37 °C with X002 or the comparison drug at the indicated concentrations. After 4 h of culture, GVBD was compared between drugs at the same concentration (*, P < 0.05 according to 2-way ANOVA).

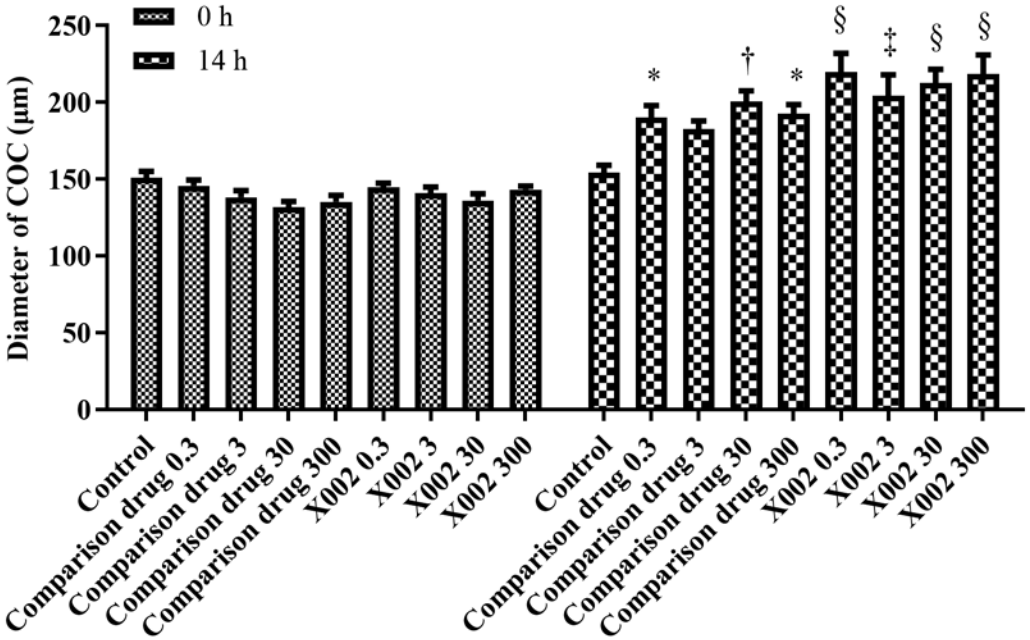

Figure 3. Diameters of cumulus-oocyte complexes (COC; n = 80 to 100 per group) before (0 h) and after 14 h of treatment with X002 or the comparison drug at the indicated concentrations (in nmol/L). COC diameters were measured by using Image J software; 2-way ANOVA was used to compare diameters (*, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; §, $P < 0.0001$ relative to untreated controls). COC diameter did not differ significantly between treatment groups at the same dose.

short-acting comparison agent (approximately 7 h in Sprague–Dawley rats).[15]

Effect of X002 on rat ovarian weight. The ratio of ovarian weight to body weight gradually increased as the dose of X002 increased, showing positive dose correlation (Figure 7). Compared with the ratios in the untreated control group, this ratio was significantly higher in rats treated with the short-acting comparison agent ($P < 0.05$) and in those given 3, 6, or 12 pmol X002 ($P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively; Figure 7). At 3 pmol, the ovarian weight:body weight ratio of the X002 group was not significantly higher than that of rats given the comparison agent ($P > 0.05$).

As compared with untreated control rats, serum estradiol levels (Figure 8) were significantly higher in rats treated with either the comparison agent ($P < 0.05$) or with 3, 6, or 12 pmol of X002 ($P < 0.01$, $P < 0.01$, and $P < 0.001$, respectively). At 3 pmol, the estradiol levels in X002-treated rats was not significantly higher than that of rats given the short-acting comparison drug. In addition, regardless of the dosage or drug, progesterone levels (Figure 9) were significantly ($P < 0.001$) higher in rats treated X002 or the comparison agent as compared with the untreated control group. The progesterone content was similar between rats treated with 3-pmol X002 and those treated with the comparison agent.

Superovulation in rat ovaries. As compared with the untreated control group, the numbers of ovulated oocytes gradually increased as the X002 concentration increased, showing positive dose correlation (Figure 10). The 3-pmol dose of the comparison drug and the 12-pmol dose of X002 both significantly ($P < 0.001$) increased the number of ovulated oocytes. However, rats in the 3-pmol X002 treatment group ovulated fewer ($P < 0.001$) oocytes than those that received 3 pmol of the comparison agent.

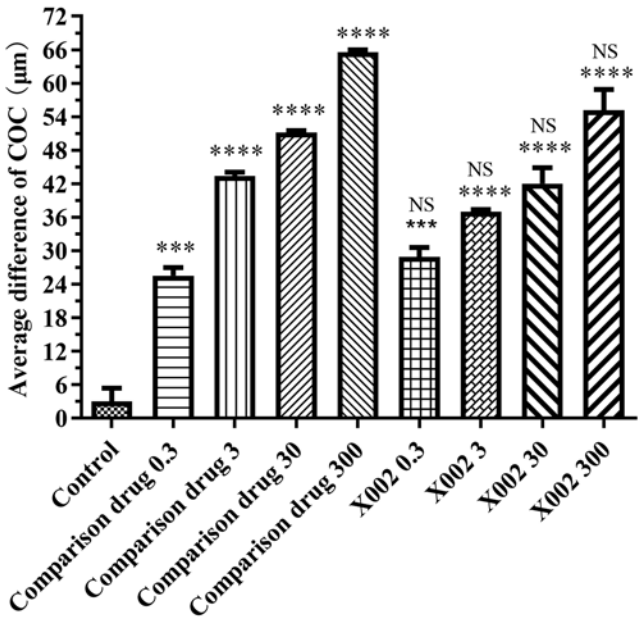

Figure 4. Comparison of the diameters of COC (n = 80 to 100 per group) before (0 h) and after 14 h of treatment with X002 or the comparison FSH drug at the indicated concentrations (in nmol/L). Average COC expansion = average COC diameter at 14 h – average COC diameter at 0 h. 1-way ANOVA was used to compare diameters (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ relative to untreated controls; NS (no significant difference), $P > 0.05$ relative to group comparison drug at the same concentrations). The experiment was performed in twice with similar results and the data have been combined.

Discussion

Several FSH-related biologic agents are used currently for assisted reproduction in human clinical practice. In addition, urine-derived FSH products, such as urofollitropin, have been used in China. Although these products are inexpensive and

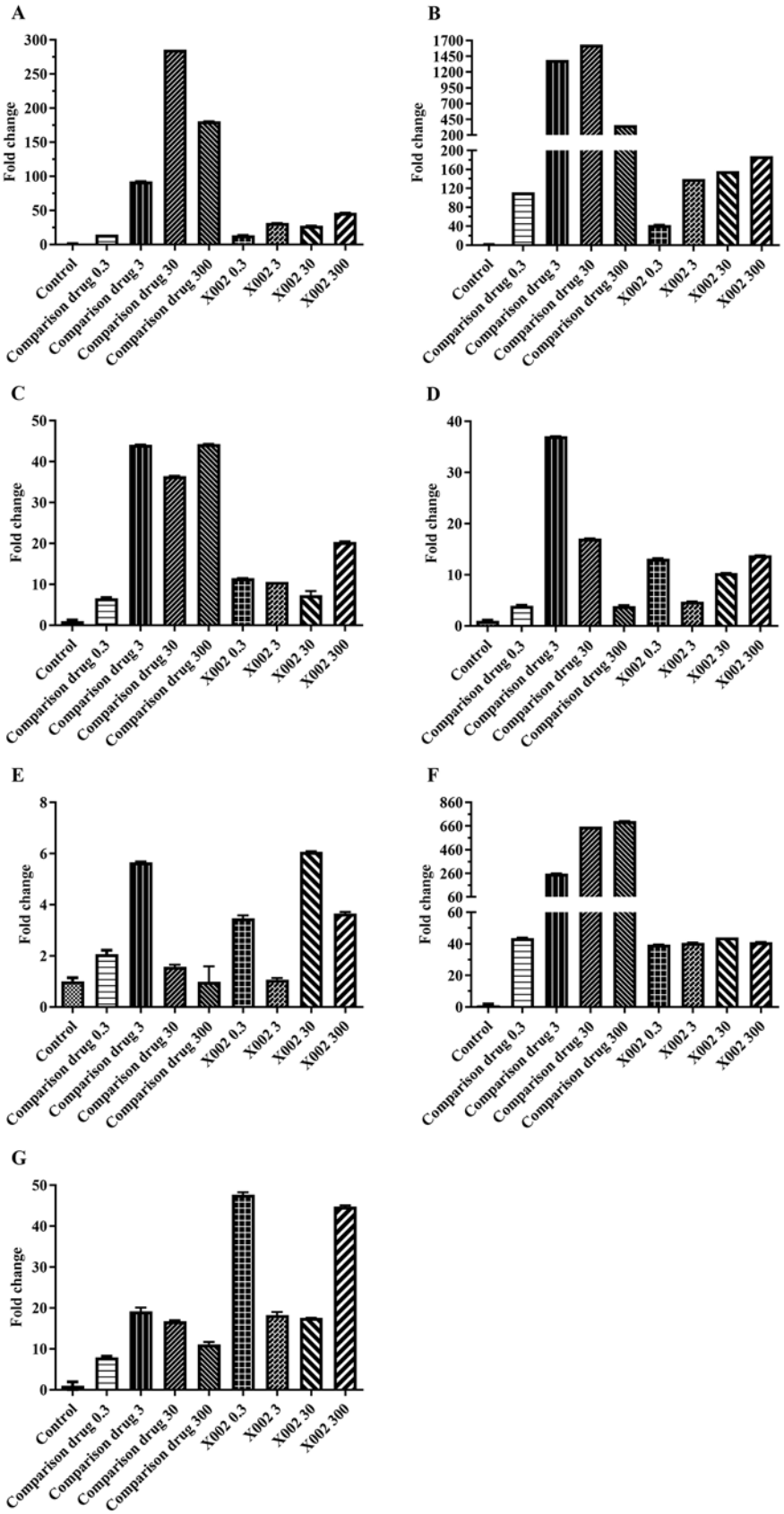

Figure 5. Expression levels of genes related to COC expansion after treatment with X002 or the comparison FSH drug at the indicated concentrations. Gene expression levels were determined through RT-qPCR analysis and normalized to endogenous β-actin mRNA levels; fold change was determined by setting the relative transcript level of the control sample to 1.[11] COC (n = 80–100 per group) were treated with the comparison FSH-like drug (0.3 nmol/L) or X002 (0.3, 3.0, 30.0, or 300.0 nmol/L) for 14 h. Differences in expression level relative to the untreated control were determined through one-way ANOVA. (A) Amphiregulin. (B) Epiregulin. (C) Betacellulin. (D) Pentraxin 3. (E) Hyaluronan synthase 2. (F) Prostaglandin-endoperoxide synthase 2. (G) Tumor necrosis factor-inducible gene 6.

Table 2. Pharmacokinetic parameters in rats after a single subcutaneous injection of X002 (mean ± 1 SD, n = 8)

		X002 dose		
	Unit	5 μg/kg	15 μg/kg	45 μg/kg
Elimination rate constant	1/h	0.003[b]	0.004 ± 0.001	0.005 ± 0.001
$t_{1/2}$	h	262[b]	181 ± 35	140 ± 34
T_{max}[a]	h	72–168	48–120	72–120
C_{max}	ng/mL	31 ± 9	82 ± 13	230 ± 29
$AUC_{(0\text{-}t)}$	h × ng/mL	7977 ± 1533	20645 ± 2510	62353 ± 5579
$AUC_{(0\text{-}inf)}$	h × ng/mL	12300 ± 4393	22342 ± 2558	64986 ± 5817
$AUC_{(t\text{-}inf)}$%	%	12.6[b]	7.6 ± 2.3	4.0 ± 2.1
Volume of distribution	mL/kg	158[b]	178 ± 37	140 ± 34
Clearance	mL/h/kg	0.42[b]	0.68 ± 0.07	0.70 ± 0.06
Mean residence time	h	322	260 ± 29	230 ± 16

[a]Provided as a range
[b]Serum dug concentrations that were outside of the mean ± 3 SD were excluded. (n = 1 or 2)

effective in assisted reproduction, frequent injections are required. Long-acting FSH products with relatively long half-lives require fewer injections. Currently, long-acting FSH-related products are generated mainly through the addition of glycosylate sites to the α and β subunits of natural FSH or through fusion of FSH subunits with the Fc segment of IgG.[9] In one commercial agent (Elonva), the β subunit C-terminal fragment of hCG is fused to the β subunit of natural FSH, thus increasing the number of glycosylation sites on FSH and ultimately extending the half-life of the product as compared with natural FSH.[3] In 2010, Elonva was officially approved by the European Medicines Agency and thus became the first long-lasting FSH product worldwide. In an assisted reproductive cycle, it is injected once and lasts for approximately 7 d, but supplementation with short-acting FSH products is necessary.[12] Another product (KN015, New drug code: LM001) prolongs the drug half-life by fusing the Fc segment of human IgG1 to the β subunit of natural FSH; only a single injection of this agent will likely be required during an assisted reproductive cycle.[14] KN015 has been in clinical trials since 2016 and is currently in phase III trials (China clinical trial registration number, CTR20181723).

The mode of action and signaling pathways of X002 may differ from those of natural FSH, perhaps due to the X002 protein structure. X002 (China patent number: CN106496331B) is a fusion between the Fc segment of human IgG4 and the α and β subunits of natural FSH; it generates relatively little antibody-dependent cell-mediated cytotoxicity and few complement-dependent cytotoxic effects in vivo (China patent number: CN106496331B). Our data show that X002 significantly promotes the expansion of mouse COCs and upregulates the expression of key genes for oocyte maturation. In terms of pharmacology, X002 promotes the maturation of mouse oocytes (Figure 3, Figure 4, and Figure 5 [A, B, C, D, E, F, G]) and significantly promotes ovarian weight gain (Figure 7) and superovulation (Figure 10) in immature rats. The bioactivity of X002 was similar to that of the comparison drug, although X002 had a more modest treatment effect (Figure 2, Figure 7, Figure 8, and Figure 9). However, X002 had a much longer half-life (greater than 140 ± 34 h) than do commercially available short-acting agents (for example, approximately 7 h (Gonalf) or 84 h (KN015) in Sprague-Dawley rats[15] and 17.3 ± 0.4 h (Elonva) in Wistar rats[13]). Owing to its technology, X002 has high

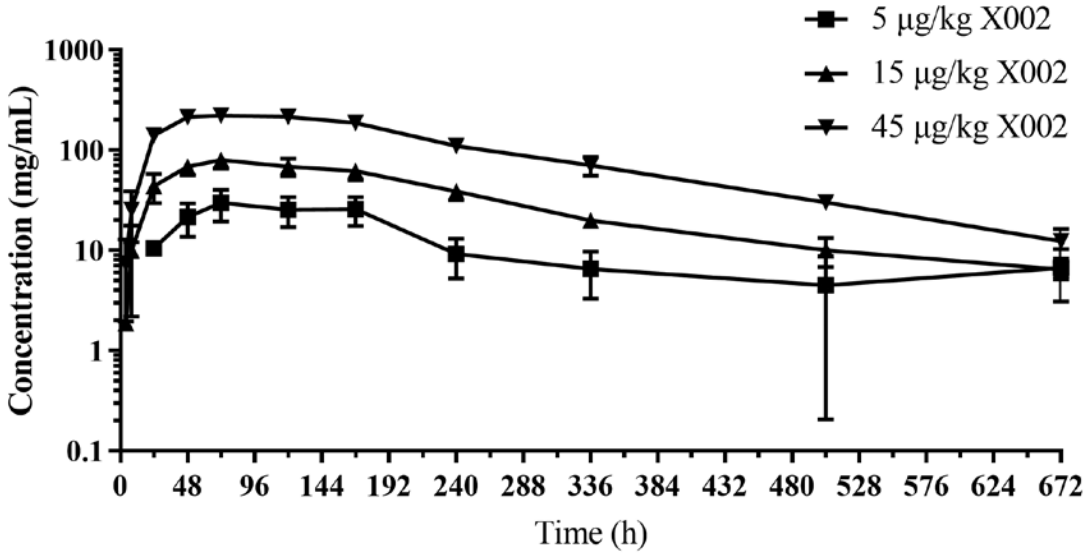

Figure 6. Average blood concentration time curve in rats after a single subcutaneous injection of X002 (5, 15, or 45 μg/kg).

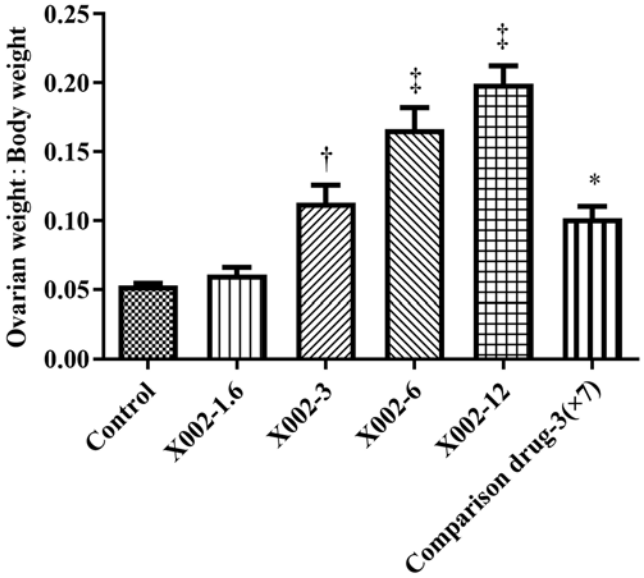

Figure 7. Ratio of ovarian weight to body weight in rats. Rats were injected subcutaneously with X002 (single injection of 1.6, 3, 6, or 12 pmol) or the comparison FSH drug (3 pmol per injection, 7 injections, with 12 h between injections), followed by 3.75 IU hCG 84 h later. At 12 h after hCG treatment, ovary and body weights were measured. One-way ANOVA was used to determine significant differences between values (*, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$ relative to untreated controls). Ratios did not differ significantly between drugs at the same concentration.

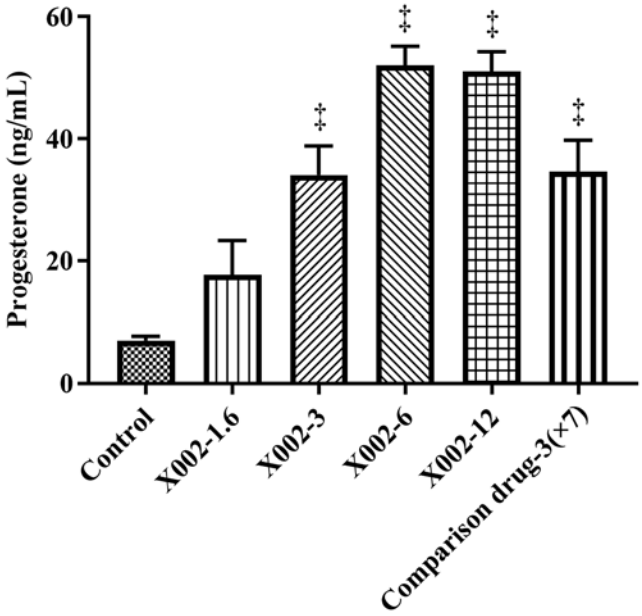

Figure 9. Serum progesterone level in rats at 96 h after the first administration of an FSH-like drug. Rats were injected subcutaneously with X002 (single injection of 1.6, 3, 6, or 12 pmol) or the comparison FSH-like drug (3 pmol per injection, 7 injections, with 12 h between injections), followed by 3.75 IU hCG 84 h later. At 12 h after hCG treatment, blood samples were collected. Serum progesterone concentrations were measured by using a rat progesterone ELISA kit. One-way ANOVA was used to determine significant differences between values (*, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$ relative to untreated controls). Progesterone levels did not differ significantly between drugs at the same concentration.

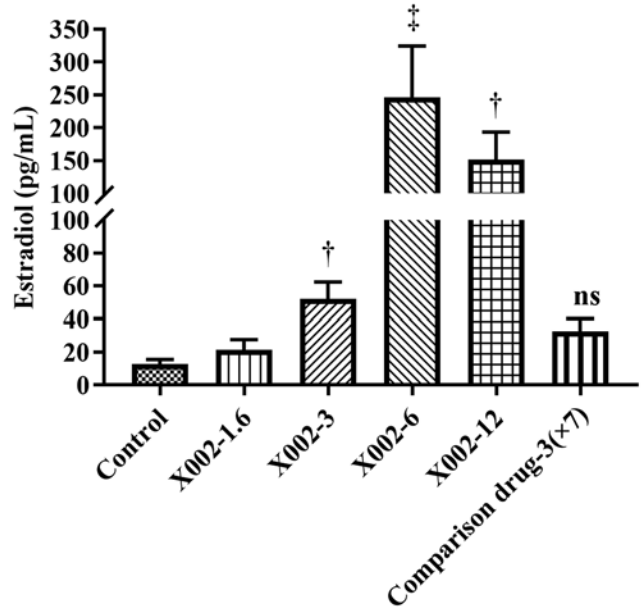

Figure 8. Serum estradiol level in rats at 96 h after the first administration of an FSH-like drug. Rats were injected subcutaneously with X002 (single injection of 1.6, 3, 6, or 12 pmol) or the comparison FSH-like drug (3 pmol per injection, 7 injections, with 12 h between injections), followed by 3.75 IU hCG 84 h later. At 12 h after hCG treatment, blood samples were collected. Serum estradiol concentrations were measured by using a rat estradiol ELISA kit. One-way ANOVA was used to determine significant differences between values (*, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$ relative to untreated controls). Estradiol levels did not differ significantly between drugs at the same concentration.

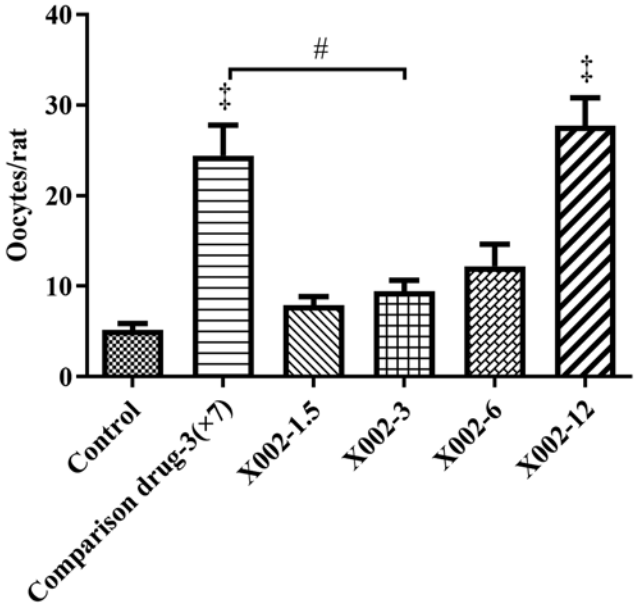

Figure 10. Number of ovulated oocytes in rats at 108 h after X002 treatment. Superovulation was stimulated through the subcutaneous administration of X002 (single injection of 1.6, 3, 6, or 12 pmol) or the comparison FSH-like drug (3 pmol per injection, 7 injections, with 12 h between injections), followed by induction with 20 IU of hCG at 84 h after the first drug administration; oocytes were counted at 24 h after hCG injection. One-way ANOVA was used to determine significant differences between values: *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$ relative to untreated controls; +, $P < 0.05$; ×, $P < 0.01$; #, $P < 0.001$ relative to same dose of the comparison drug).

target-product yield and low production of heterodimeric by-products (China patent number: CN106496331B).

In conclusion, X002 showed bioactivity similar to that of the short-acting comparison agent in terms of the superovulation, development, and maturation of oocytes and thus is potentially suitable for promoting the production of numerous mature and healthy egg cells in assisted reproduction protocols. We have not yet compared the pharmacokinetic and pharmacodynamic properties of X002 with those of a commercially available FSH agents with a long half-life (for example, Elonya), but we plan to do this in follow-up studies. A phase I study of X002 is currently open (China clinical trial registration number: CTR20210824). In the future, X002 may benefit patients seeking assisted human reproduction.

Acknowledgments

We thank United Power Bioengineering and Biotechnology (Beijing, China) for providing X002 and the United Power Pharma Tech (Beijing, China) for providing the pharmacodynamic research platform. We also thank Professors Haoshu Luo and Hua Zhang and Dr Ye Wang (China Agricultural University, Beijing, China) for providing theoretical and technical support for the pharmacodynamic research. The study was supported by Beijing Qikang Xingye Biopharma Technology (grant no. X002).

References

1. **American Veterinary Medical Association.** 2020. AVMA guidelines for the euthanasia of animals, 2020 edition. Schaumburg (IL): American Veterinary Medical Association. Available at https://www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf.
2. **Choi JK, He X.** 2015. Improved oocyte isolation and embryonic development of outbred deer mice. Sci Rep **5**:12232. 10.1038/srep12232.
3. **Croxtall JD, McKeage K.** 2011. Corifollitropin alfa: A review of its use in controlled ovarian stimulation for assisted reproduction. BioDrugs **25**:243–254. 10.2165/11206890-000000000-00000.
4. **He TP, Tang JY, Xu L, Song CL, Lei T.** 2011. Expression of recombinant human follicle-stimulating hormone in cho cells. Chinese J Biologicals **24**:1409–1412.
5. **Jung S, Park Y, Kim Y, Kim YY, Choi H-J, Son W-C, Kwon S.** 2014. LAPS-FSH: A new and effective long-acting follicle-stimulating hormone analogue for the treatment of infertility. Reprod Fertil Dev **26**:1142–1153. 10.1071/rd13118.
6. **Leibowitz D, Hoffman J.** 2000. Fertility drug therapies: Past, present, and future. J Obstet Gynecol Neonatal Nurs **29**:201–210. 10.1111/j.1552-6909.2000.tb02040.x.
7. **Loutradis D, Drakakis P, Vlismas A, Antsaklis A.** 2009. Corifollitropin alfa, a long-acting follicle-stimulating hormone agonist for the treatment of infertility. Curr Opin Investig Drugs **10**:372–380.
8. **Low SC, Nunes SL, Bitonti AJ, Dumont JA.** 2005. Oral and pulmonary delivery of FSH-Fc fusion proteins via neonatal Fc receptor-mediated transcytosis. Hum Reprod **20**:1805–1813. 10.1093/humrep/deh896.
9. **Meduri G, Bachelot A, Cocca MP, Vasseur C, Rodien P, Kuttenn F, Touraine P, Misrahi M.** 2008. Molecular pathology of the FSH receptor: new insights into FSH physiology. Mol Cell Endocrinol **282**:130–142. 10.1016/j.mce.2007.11.027.
10. **Pouwer AW, Farquhar C, Kremer JAM.** 2012. Long-acting FSH versus daily FSH for women undergoing assisted reproduction. Cochrane Database Syst Rev **1**:CD009577. 10.1002/14651858.cd009577.pub2
11. **State Pharmacopoeia Commission of the People's Republic of China.** 2015. Pharmacopoeia of the People's Republic of China, volume **1**, 10th edition. Beijing: China Medical Science Press.
12. **Tsakiridis I, Najdecki R, Tatsi P, Timotheou E, Kalinderi K, Michos G, Virgiliou A, Yarali H, Athanasiadis A, Papanikolaou EG.** 2020. Evaluation of the safety and efficacy of corifollitropin alfa combined with GnRH agonist triggering in oocyte donation cycles. A prospective longitudinal study. JBRA Assist Reprod **24**:436–441. 10.5935/1518-0557.20200033.
13. **Verbost P, Sloot WN, Rose UM, de Leeuw R, Hanssen RG, Verheijden GF.** 2011. Pharmacologic profiling of corifollitropin alfa, the first developed sustained follicle stimulant. Eur J Pharmacol **651**:227–233. 10.1016/j.ejphar.2010.10.078.
14. **Xu T, Guo KP, Wang JJ, Wu J, Ji JY, Huang Y, Xu T.** 2015. FSH (follicle-stimulating hormone) fusion protein, and preparation method and application thereof. China patent: CN103509121B.
15. **Zhang YL, Guo KP, Ji SY, Liu XM, Wang P, Wu J, Gao L, Jiang TQ, Xu T, Fan HY.** 2016. Development and characterization of a novel long-acting recombinant follicle stimulating hormone agonist by fusing Fc to an FSH-β subunit. Hum Reprod **31**:169–182. 10.1093/humrep/dev295.