A novel long-acting, follicle stimulating hormone-Fc fusion protein displays Gonal-f-like function *in vitro* and *in vivo*

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Abstract

Purpose

To explore whether X002 can enhance bioactivity and has a long half-time *in vitro* and *in vivo*.

Methods

For the *in vitro* study, GVBD rate and COC expansion were applied. In the GVBD test, 21–24-day-old female KM mice were stimulated with PMSG for 46 h, and the naked oocytes of ovaries were then collected. Four hours after X002 treatment at 37°C, the GVBD rates of naked oocytes were counted. In COC expansion, COCs were collected from mice stimulated with PMSG. After coculture of COCs and X002 for 14 h, COC diameter was measured, and the expression of genes involved in COC expansion was also determined by RT-qPCR. For the *in vivo* study, 6–8-week-old female SD rats were administered a subcutaneous injection of X002 for pharmacokinetics. Serum was assayed by ELISA at different time points. For pharmacodynamics, 26-day-old female SD rats were used in ovarian weight and superovulation assay. To assay ovarian weight, rats were stimulated with hCG 84 h after X002 treatment. At 12 h after hCG injection, ovaries were weighted and E2 or P4 in serum was quantified. To assay superovulation, rats were treated with the above methods. At 108 h after X002 treatment, oocytes were counted from fallopian tubes.

Results

X002 promoted GVBD and COC expansion *in vitro* and effectively promoted significant ovarian weight gain and superovulation, similar to Gonal-f; Meanwhile, it had a longer $T_{1/2}$.

Conclusions

X002 is a long-acting FSH-liked agent which has good bioactivity, similar to Gonal-f.

Introduction

Follicle-stimulating hormone (FSH) is a glycoprotein secreted by the gonadotropic cells of the anterior pituitary gland [1]. The main physiological function of FSH is to promote the development and maturation of female follicles, to support sertoli cell functions in male. FSH is composed of two subunits, α and β. In addition to GnRH (Gonadotropin-releasing hormone) agonist or antagonist, FSH is mainly used for assisted reproduction in clinical practice, especially recombinant FSH [2]. The main purpose of FSH-related products is to produce more mature oocytes with the help of ovulation-induction medications, such as hCG (human chorionic gonadotropin). Currently in the process of assisted reproduction, patients need to inject short-acting FSH-related drugs every day, or long-acting FSH-related

drugs once as a single injection. The FSH-related products currently on the market are recombinant hormones, such as Gonal-f, Puregon and Elonva (Corifollitropin alfa), or urine-derived FSH. As a short-acting FSH-related drug, Gonal-f is given every day, while Elonva is given once as a single injection under the skin, which is sufficient for the first 7 days of OS period, as it has longer half-life. According to the Elonva instruction manual, seven days after the Elonva injection, if further ovarian stimulation is needed, injections of another medicine similar to Elonva, but suitable for daily injection, may be given, such as Gonal-f (https://www.drugs.com/uk/elonva.html). Although these FSH products are safety and similar to the natural (endogenous) FSH, and meet the requirement of assisted reproductive technology to a certain extent on the market, they lead to insufficient efficacy, poor patient compliance or high cost.

For solving insufficient efficacy and poor patient compliance, the fusion of FSH two subunits with human IgG1 FC (single chain FSH-FC or heterodimer FSH-FC) [3], the C-terminal fusion of FSH-β subunit (the human chorionic gonadotropin (hCG) carboxyl-terminal peptide (CTP)) (Elonva) [4], and the fusion of FSH with human IgG4 FC (LAPS-FSH) [5] was often used. Despite these products are in clinical trials or on sale, they still can't satisfy the market's demand completely. Facing the huge market of assisted reproduction, we also used genetic engineering technology to fuse the Fc segment of human immunoglobulin G4 (IgG4) with FSH (code: X002; molecular weight = ~90 kDa). In this study, we explored whether X002 has a higher bioactivity and a longer half-time than Gonal-f.

Materials And Methods

Major drugs and reagents

FSH-Fc fusion protein (code: X002; molecular weight = ~90 kDa) is a new type of protein produced by fusing the Fc fragment of the human IgG4 subtype with α and β subunits of FSH (Figure 1). It was commissioned by the United Power Bioengineering and Biotechnology (Production Batch No. X0020220171205; Beijing, China) and developed by Beijing Qikang Xingye Biopharma Technology Co., Ltd. (Beijing, China). Gonal-f was purchased from Merck Serono (specification: 5.5 μg, 75 IU; Darmstadt, Germany), pregnant mare serum gonadotropin (PMSG) was purchased from Beijing Solarbio Science and Technology (Product No. P9970; Beijing, China), and human chorionic gonadotrophin (hCG) was purchased from ProSpec-Tany TechnoGene Ltd. (Product No. hor-250; Rehovot, Israel).

Major equipment and instruments

The major equipment and instruments used in this study included the Roche Cobas®4000 automatic analyser (Model No. Cobas®4000; Roche Diagnostics, Shanghai, China), the Stereo Microscope (Model No. SMZ800N; Nikon Imaging Instrument Sales, Beijing, China), an ordinary scale (Model No. YP2002; Shanghai YuePing Scientific Instrument (Suzhou) Manufacturing, Jiangsu Province, 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 26-day-old, specific-pathogen free (SPF) grade female Sprague Dawley (SD) rats, 6–8-week-old female SD rats (SPF grade), and 21–24-day-old female Kun Ming (KM) mice (SPF grade) purchased from Beijing HFK Bioscience Co., Ltd (Experimental Animal Production License No. SCXK (Beijing) 2014-0004; Beijing, China). The SD rats and KM mice were housed in SPF-grade rooms under a constant temperature of 20–26 °C, constant humidity of 40–70%, and alternating 12:12 h light:dark cycle. Animals had free access to food and water. All animal experiments were approved by the Committee of Animal Experiments and Experimental Animal Welfare of Beijing Qikang Xingye Biopharma Technology Co., Ltd. (Grant No.: IACUC2018-0002), and in strict accordance with the 3R principle (Reduction, Replacement, Refinement).

Detection of germinal vesicle breakdown (GVBD) in mouse oocytes

The 21–24-day-old female KM mice were subcutaneously injected with PMSG (5 IU/animal), followed by euthanisation with CO_2 at 46 h after injection to collect the ovaries, and isolate and process the cumulus-oocyte complexes (COCs) to eventually obtain the naked oocytes. The oocytes (n = 80–100 per group) were added to a culture medium containing Gonal-f or X002 at concentrations of 0.3, 3.0, 30.0, or 300.0 nmol/L [6] for 4 h at 37 ℃, after which the GVBD rates of the oocytes were compared between the same concentrations of Gonal-f and X002 [3]. The GVBD rate was counted according to the following equation: GVBD rate (%) = (the number of oocytes in GVBD phase + the number of oocytes in first polar body phase) / (total number of oocytes − number of dead and degenerated oocytes) × 100%.

Analysis of mouse COC expansion

The 21–24-day-old female KM mice were subcutaneously injected with PMSG (5 IU/animal) and euthanised with CO_2 at 46 h after injection to collect the ovaries and isolate the COCs. The COCs (n = 80–100 per group) were then incubated with culture medium containing Gonal-f or X002 at concentrations of 0.3, 3.0, 30.0, or 300.0 nmol/L[6] for 14 h at 37℃, after which the expanded diameters of COCs were compared at the same concentrations of Gonal-f and X002. The total RNA of COCs in the different groups was extracted according to the manufacturer instructions of the RNAsimple Total RNA Kit (catalog number: DP419, TIANGEN Biotech (Beijing)Co.,Ltd), and reverse transcription was used to synthesize the cDNA according to the manufacturer's instructions of the revertaid first strand cDNA synthesis kit (catalog number: K1622, Thermo Fisher Scientific Inc). The cDNA templates of different COC groups were used for RT-qPCR to measure the expression of key genes related to the maturation process of oocytes, namely amphiregulin (*Areg*), epiregulin (*Ereg*), betacellulin (*Btc*), pentraxin 3 (*Ptx3*), hyaluronan synthase 2 (*Has2*), prostaglandin-endoperoxide synthase 2 (*Ptgs2*), and tumour necrosis factor-inducible gene 6 (*Tnfaip6*), according to the instructions of AceQ qPCR SYBR Green Master Mix (catalog number: Q121-02, Vazyme Biotech Co.,Ltd) and using the primers of individual genes (Table 1) [6].

The individual gene expression levels were determined and normalised to the internal reference gene, β-actin. The relative expression level of each gene in the control group was set to 1. The $2^{(-\Delta\Delta Ct)}$ method

was used to calculate the relative expression level (fold-change) of the corresponding gene in the experimental group.

Pharmacokinetic tests

To evaluate the pharmacokinetics (PKs) of X002, female SD rats (n = 8 per group) were administered a subcutaneous injection of X002 (5, 15, or 45 μg/kg), and the peripheral blood was collected from the orbital venous plexus at 4, 8, 24, 48, 72, 120, 168, 240, 336, 504, and 672 h after X002 administration. The collected peripheral blood samples were coagulated and centrifuged at 2,000 ×g for 20 min at 4 °C before the rat serum was separated. The rat serum samples were analysed by enzyme-linked immunosorbent assay (FSH enzyme immumoassay test kit, minimum sensitivity: 2.5 mIU/ml, catalog number: BC-1029, BioCheck, Inc.) for X002 at different concentrations. The WinNonLin® v 6.4 (Certara Corp.) software was used to calculate the PK parameters.

Testing the rat ovarian weight gain

Following the guidelines of the Pharmacopoeia of the Peoples' Republic of China (2015 edition), 26-day-old female SD rats were chosen to test acting function of X002. After randomly grouping the 26-day-old female SD rats (n = 10 per group) according to their body weight, the animals were subcutaneously injected with 3.0 pmol per (each animal * each injection) Gonal-f (7 injections, with an interval of 12 h between each injection), 1.6 pmol per (each animal * each injection) X002 (single injection), 3.0 pmol per (each animal * each injection) X002 (single injection), 6.0 pmol per (each animal * each injection) X002 (single injection), or 12.0 pmol per (each animal * each injection) X002 (single injection)[6]. 84 h after X002 treatment, all animals were also injected subcutaneously with 3.75 IU per (each animal * each injection) hCG (n = 10 rats per group). The rats were euthanised with CO_2 at 96 h after the first administration of corresponding treatments to collect their venous blood samples, which were subjected to coagulation, followed by dissecting and weighing the ovaries. The estradiol (E2) and progesterone (P4) levels were quantified by rat E2 or P4 ELISA kit (Estradiol (rat) Elisa kit, catalog number: K3831, BioVision, Inc.; rat progesterone Elisa kit, catalog number: 80558, Crystal Chem Inc.), respectively.

Detection of superovulation in rats

After randomly grouping the 26-day-old female SD rats (n = 10 per group) according to their body weight, the animals were subcutaneously injected with 3.0 pmol per (each animal * each injection) Gonal-f (7 injections in total, with an interval of 12 h between two injections), 1.6 pmol per (each animal * each injection) X002 (single injection), 3.0 pmol per (each animal * each injection) X002 (single injection), 6.0 pmol per (each animal * each injection) X002 (single injection), or 12.0 pmol per (each animal * each injection) X002 (single injection)[6]. They were all subcutaneously injected with 20 IU per (each animal * each injection) hCG 84 h after the first administration of the corresponding treatment (n = 10 rats per group). The rats were euthanised with CO_2 108 h after the first administration of corresponding treatments to isolate the rat fallopian tubes for the counting of oocyte numbers inside [6].

Statistical analysis

All data are presented as the mean ± standard error of the mean or mean ± standard deviation. GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used for graph preparation, and the SPSS v22.0 software (IBM, Armonk, NY, USA) was used for one-way analysis of variance (one-way ANOVA) and two-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

X002 promotes an increase in GVBD rate in mouse oocytes

The oocytes of each group were incubated with a culture medium containing Gonal-f or X002 at gradient concentrations of 0.3, 3.0, 30.0, or 300.0 nmol/L for 4 h before calculating the GVBD rate of the oocytes (Figure 2). The GVBD rate of the oocytes gradually increased as the Gonal-f or X002 concentration increased. The GVBD rate of the oocytes treated with 0.0–30.0 nmol/L X002 was equivalent to that of the oocytes treated with 0.0–30.0 nmol/L Gonal-f. When the concentration of X002 was increased to 300.0 nmol/L, the GVBD rate of the oocytes was lower than that of the Gonal-f group at the same concentration ($P < 0.05$), and similar to that of the X002 at 30.0 nmol/L. These results showed that X002 is able to promote GVBD in mouse oocytes, and similar to Gonal-f at 0.0–30.0 nmol/L, but more than 30 nmol/L, X002 was saturated effect, and lower than Gonal-f.

X002 promotes mouse COC expansion

After incubating COCs in a culture medium containing Gonal-f or X002 at gradient concentrations of 0.3, 3.0, 30.0, or 300.0 nmol/L for 14 h, the COC diameters of the different groups were measured (Image J software was applied), and the average differences in COC diameter before and after COC expansion were calculated. Figure 3 and Figure 4 shows the changes in COC diameter before and after treatment with different concentrations of Gonal-f or X002. Compared to the control group, the COC diameter in the X002 group was significantly increased at 14 h after the corresponding treatments ($P < 0.01$). Figure 5 shows the differences in average COC diameters before and after the expansion. Compared to the control group, the differences in average COC diameters of the X002 groups were significantly increased. The differences in average COC diameters of the X002 groups at 0.3 nmol/L and 3.0 nmol/L were greater than those of the Gonal-f groups at the corresponding concentrations. However, the differences in average COC diameters of the X002 groups at 30.0 and 300.0 nmol/L were smaller than those of the Gonal-f groups at the corresponding concentrations. RT-qPCR was used to measure the expression levels of the key genes *Areg*, *Ereg*, *Btc*, *Has2*, *Ptgs2*, *Tnfaip6*, and *Ptx3* during oocyte maturation. As shown in Figure 6, compared to the control group, oocytes after Gonal-f or X002 treatment had significantly upregulated expression of key genes in different groups. Compared to the Gonal-f group, expression of *Areg*, *Ereg*, and *Ptgs2* in the oocytes of the X002 treatment groups had no significant changes, whereas expression of *Btc*, *Ptx3*, *Has2*, and *Tnfaip6* in the oocytes of the X002-treated groups changed significantly. However, no dose-dependent correlation was found with X002 treatment. X002 significantly promotes the expansion of mouse COCs and upregulates the expression of key genes for oocyte

maturation. The mode of action and signalling pathways activated by X002 may be different from traditional FSH and may be related to changes in X002 protein structure.

X002 has a relatively long $T_{1/2}$

After a single subcutaneous administration of different doses of X002, the peripheral blood samples of rats were collected at different time points to collect the serum for the detection of X002 concentration in the serum samples and analyse PK parameters of X002. As shown in Table 2 and Figure 7, the peak time (T_{max}) of X002 at different was were 72–168 h, 48–120 h, and 72–120 h, the $T_{1/2}$ of X002 at different doses was 262.43 h, 181.24 ± 34.59 h, and 139.81 ± 33.90 h, the peak concentrations (C_{max}) of X002 at different doses were 31.12 ± 9.47 ng/mL, 81.85 ± 12.97 ng/mL, and 230.06 ± 28.76 ng/mL, and the system exposure level [area under the curve (0-t)/AUC(0-t)] was 7976.57 ± 1533.16 h*ng/mL, 20645.02 ± 2510.23 h*ng/mL, and 62352.89 ± 5578.88 h*ng/mL. The peak concentration ratio of different doses of X002 treatment was 1:2.6:7.4, the AUC(0-t) ratio was 1:2.6:7.8, and the dosage ratio of X002 was 1:3:9. The ratio of AUC(0-t) was similar to the dose ratio, basically showing linear PK characteristics. These results indicate that X002 has a longer $T_{1/2}$ than Gonal-f (around 7h in SD rats) (Zhang et al., 2016).

X002 promotes an increase in rat ovarian weight

Comparisons of the ovarian weight of the rats in different groups after a 96-h treatment of Gonal-f or X002 at different doses showed that the ratio of ovarian weight to body weight gradually increased as the dose of X002 increased, showing a positive dose-correlation. The ratio of ovarian weight to body weight of the Gonal-f-treatment group was significantly higher than that of the control group ($P < 0.05$), and the ratio of ovarian weight to body weight of the 3, 6, and 12 pmol X002 treatment groups was significantly higher than that of the control group ($P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively) (Figure 8). At 3 pmol, the ratio of ovarian weight to body weight of the X002 treatment group was slightly higher than that of the Gonal-f group, without statistical significance. Subsequent measurement of serum E2 levels (Figure 9) showed that E2 content was significantly higher in the Gonal-f group than in the control group ($P < 0.05$), and E2 levels in the 3, 6, and 12 pmol X002 treatment groups were also significantly higher than in the control group ($P < 0.01$, $P < 0.01$, and $P < 0.001$, respectively). At 3 pmol, the E2 content in the X002 treatment group was higher than that of the Gonal-f group, but without statistical significance. As shown in Figure 10, the P4 levels of the Gonal-f and the X002 treatment groups at different concentrations were significantly higher than those of the control group ($P < 0.001$). The P4 content in the 3 pmol X002 treatment group was similar to that of the Gonal-f treatment group. These results showed that X002 and Gonal-f equally promote a significant increase in the levels of E2 and P4 hormones in rats.

X002 promotes superovulation in rat ovaries

At 84 h after stimulating with different doses of X002 or Gonal-f, 20 IU of hCG were administered. After another 24 h, the rat fallopian tubes were dissected to isolate and count the oocytes in the fallopian tubes. Compared to the control group, the numbers of ovulated oocytes in the rats gradually increased as the X002 concentration increased, showing a positive dose-correlation (Figure 11). However, treatment

with 3 pmol Gonal-f and 12 pmol X002 significantly increased the number of ovulated oocytes ($P < 0.001$), whereas the number of ovulated oocytes in the 3 pmol X002 treatment group was significantly lower than that of the Gonal-f group (3 pmol) ($P < 0.001$). These results indicated that X002 induces significant superovulation and increases the number of ovulated oocytes in rats but to a lesser extent than Gonal-f.

Discussion

To date, Gonal-f and Puregon dominate in FSH-related biological agents that are mainly used for assisted reproduction in clinical practice. Additionally, urine-derived FSH products, such as urofollitropin, also have a place in the Chinese market. Although these products meet the requirements of assisted reproduction and are inexpensive, frequent injections are required during the assisted reproductive cycle. Long-acting FSH products with a relatively long $T_{1/2}$ have emerged to minimise the number of injections. Currently, long-acting FSH-related products mainly add glycosylate sites on the α and β subunits of natural FSH or fuse with the Fc segment of IgG [1]. In Elonva, the β subunit C-terminal fragment of hCG is fused to the β subunit of natural FSH to increase the glycosylation sites on FSH, ultimately extending the $T_{1/2}$ of the product [7]. In 2010, Elonva was officially approved in Europe and was the first long-lasting FSH product worldwide. In an assisted reproductive cycle, Elonva is injected once and lasts for approximately 7 days but it requires to be supplemented with short-acting FSH products. KN015 is another product prolonging the $T_{1/2}$ by fusing the Fc segment of human IgG1 to the β subunit of natural FSH. It is expected that only one injection is required in an assisted reproductive cycle [8]. KN015 (New drug code: LM001) has been tested in clinical trials since 2016 and at present, it is in clinical stage III (China Clinical Trial Registration No. CTR20181723, www.chinadrugtrials.org.cn).

Structurally, unlike Elonva, which is already marketed, X002 is a long-acting FSH product formed by fusion with the Fc segment of human IgG4 and has a relatively long $T_{1/2}$. Compared to KN015, the molecular structure of X002 is formed by the fusion of the α and β subunits of natural FSH with IgG4-Fc, showing relatively low antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity effects *in vivo*. In terms of pharmacology, after X002 injection, X002 promotes maturation of mouse oocytes and significantly promotes ovarian weight gain and superovulation in immature rats. The effects of X002 and Gonal-f are similar; however, compared to Gonal-f, X002 has a more modest effect. Compared to the $T_{1/2}$ of Gonal-f or KN015 (The $T_{1/2}$ of Gonal-f was 7 h, and the $T_{1/2}$ of KN015 was around 84 h in SD rats) [6] or Elonva (The $T_{1/2}$ was 17.3±0.4 h in Wistar rats) [9], X002 has a longer $T_{1/2}$ (The $T_{1/2}$ was above 139.81±33.90 h). Additionally, due to the knobs into holes (KIHs) technology, X002 shows the advantages of high target product yield and low heterodimer (by-product) production in the future.

In conclusion, compared to Gonal-f (a short-acting product), X002 has similar effect on superovulation and development and maturation of oocytes, and eventually promotes the body to produce a large number of mature and healthy egg cells, which meets the needs of assisted reproduction. For long-acting

products (such as Elonva or KN015), we only made a simple comparison from the structure and half-life, and did not perform a head-to-head comparison in other aspects, such as pharmacodynamics. But we think that X002 is a novel product with long half-life and Gonal-f equivalent effect. In follow-up studies, we will continue to thoroughly investigate the mechanism of X002, and a comparative study with other similar products.

Declarations

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Ethics approval

All animal experiments were approved by the Committee of Animal Experiments and Experimental Animal Welfare of Beijing Qikang Xingye Biopharma Technology Co., Ltd. (Grant No.: IACUC2018-0002)

Consent to participate

Written informed consent was obtained from individual or guardian participants.

Consent for publication

Written informed consent for publication was obtained from all participants.

Conflict of interest statement

All authors declare no conflict of interest.

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Tables

Table 1

Primer sequences of the target genes used in RT-qPCR [6].

Gene name	Species	Primer sequence (5'-3')	PCR product size (bp)
Areg	Mouse	F: 5'-GAGGCTTCGACAAGAAAACG-3' R:5'TTTATCTTCACACATCTCTTTATGTACAG-3'	210
Ereg	Mouse	F: 5'-GCATCCCAGGAGAATCCGAG-3' R: 5'-CTCACATCGCAGACCAGTGT-3'	197
Btc	Mouse	F: 5'-GTAGCAGTGTCAGCTCCCTG-3' R: 5'-ATGCTTGTACTGCTTGGGGC-3'	206
Has2	Mouse	F: 5'-GTTGGAGGTGTTGGAGGAGA-3' R: 5'-ATTCCCAGAGGACCGCTTAT-3'	155
Ptgs2	Mouse	F: 5'-TGTACAAGCAGTGGCAAAGG-3' R: 5'-CCCCAAAGATAGCATCTGGA-3'	230
Tnfaip6	Mouse	F: 5'-TTCCATGTCTGTGCTGCTGGATGG-3' R: 5'-AGCCTGGATCATGTTCAAGGTCAAA-3'	328
Ptx3	Mouse	F: 5'-GTGGGTGGAAAGGAGAACAA-3' R: 5'-GGCCAATCTGTAGGAGTCCA-3'	190
β-Actin	Mouse	F: 5'-GCTCTTTTCCAGCCTTCCTT-3' R: 5'-GTACTTGCGCTCAGGAGGAG-3'	234

Table 2
Average pharmacokinetic parameters of rats after a single subcutaneous injection of X002 (mean ± standard deviation, n = 8)

PK parameter	Unit	X002		
		sc 5 μg/kg	sc 15 μg/kg	sc 45 μg/kg
Kel	1/h	0.003@	0.004 ± 0.001	0.005 ± 0.001
t1/2	h	262.43@	181.24 ± 34.59	139.81 ± 33.90
#T_{max}	h	72–168	48–120	72–120
C_{max}	ng/mL	31.12 ± 9.47	81.85 ± 12.97	230.06 ± 28.76
C0	ng/mL	NA	NA	NA
$AUC_{(0-t)}$	h*ng/mL	7976.57 ± 1533.16	20645.02 ± 2510.23	62352.89 ± 5578.88
$AUC_{(0-inf)}$	h*ng/mL	12299.95 ± 4393.38	22342.48 ± 2558.20	64985.56 ± 5816.53
$AUC_{(t-inf)}$%	%	12.64@	7.62 ± 2.31	4.03 ± 2.08
Vd	mL/kg	157.67@	177.56 ± 37.37	140.46 ± 34.25
CL	mL/h/kg	0.42@	0.68 ± 0.07	0.70 ± 0.06
MRT_{inf}	h	321.62	259.95 ± 29.09	229.87 ± 16.42

Note: #, using range representation; @, could not eliminate several individuals, or inaccurate parameter estimation (n = 1).

Figures

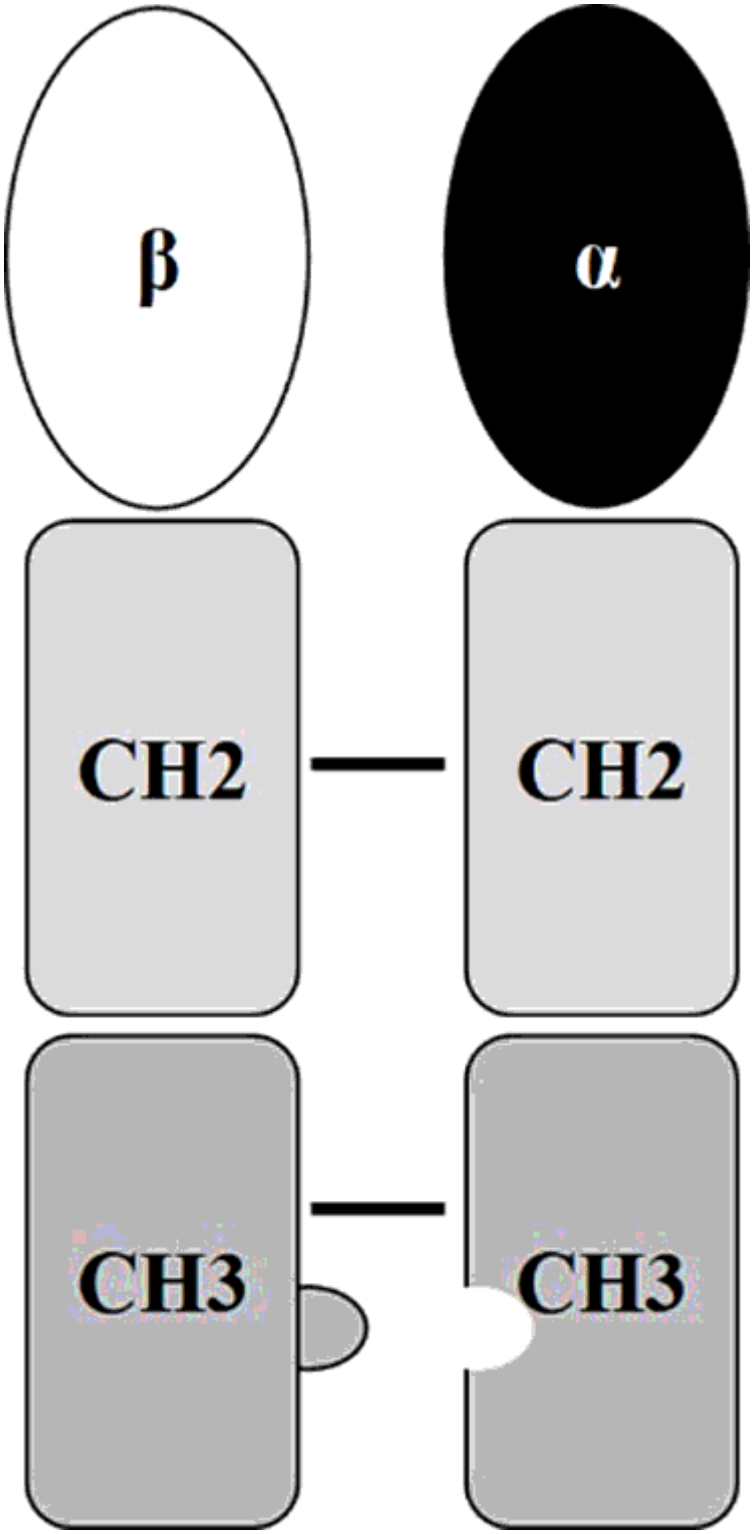

Figure 1

A schematic of the X002 fusion protein. α and β subunits of FSH were fused with the N terminal of Fc fragment of the human IgG4 subtype (containing CH2 and CH3 domains) respectively. Besides, the Fc fragment adopts an Knob into hole technique to prevent mismatches.

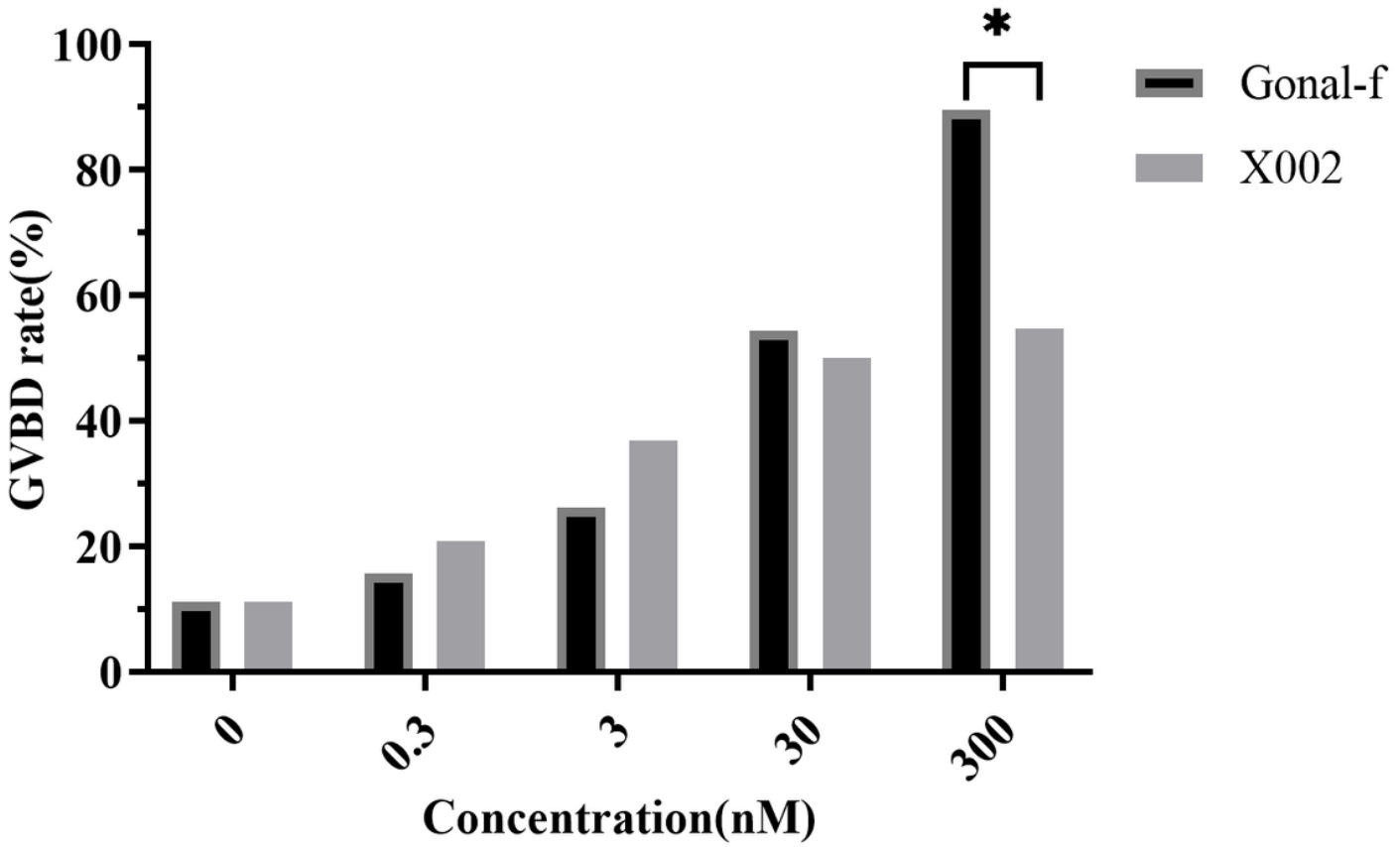

Figure 2

GVBD rates of oocytes treated with different concentrations of Gonal-f and X002. Female KM mice were subcutaneously injected with PMSG (5 IU/animal). 46 h after PMSG injection, naked oocytes were collected. And naked oocytes were cocultured with Gonal-f or X002 (0.3, 3, 30, 300 nmol/L) for 4 h at 37 °C. GVBD rates were counted after 4 h in culture (Zhang et al., 2016). Two-way analysis of variance method was used to compare between Gonal-f and X002 at same concentration (*P < 0.05).

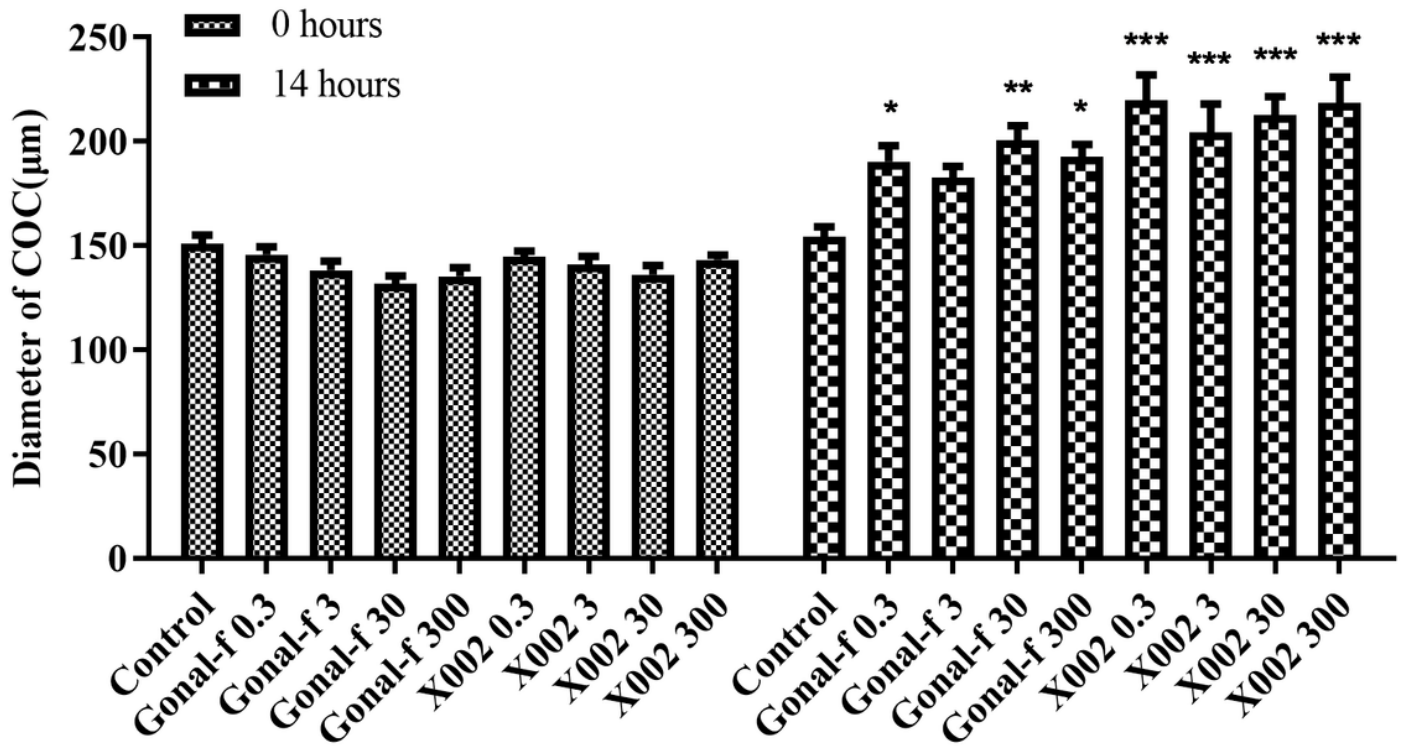

Figure 3

COC diameters after 0 and 14 h treatments with Gonal-f or X002 at different concentrations. COCs (n = 80–100 per group) were treated with Gonal-f or X002 (0.3, 3.0, 30.0, or 300.0 nmol/L) for 14 h. Image J software was applied for COC diameters. Two-way analysis of variance method was used to compare between Control, Gonal-f and X002 at same concentration. Compared to Control, *P < 0.05, **P < 0.01, ***P < 0.001. No significant difference was noted between Gonal-f and X002 at the same concentration.

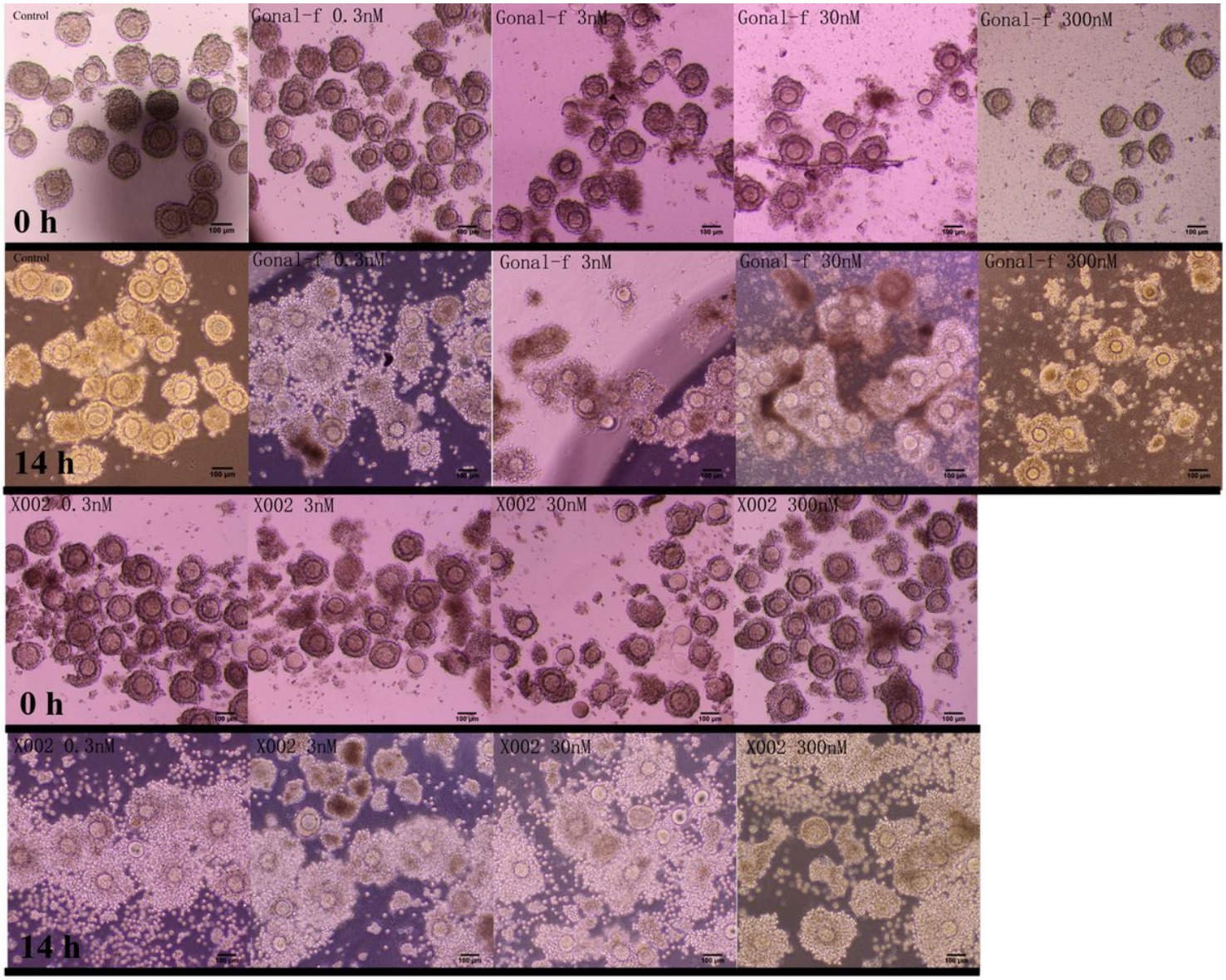

Figure 4

Average COC expansion value before and after treatment with Gonal-f or X002 at different concentrations. COCs (n = 80–100 per group) were treated with Gonal-f or X002 (0.3, 3.0, 30.0, or 300.0 nmol/L) for 14 h. Average COC expansion value = average COC diameter (14 h) - average COC diameter (0 h).

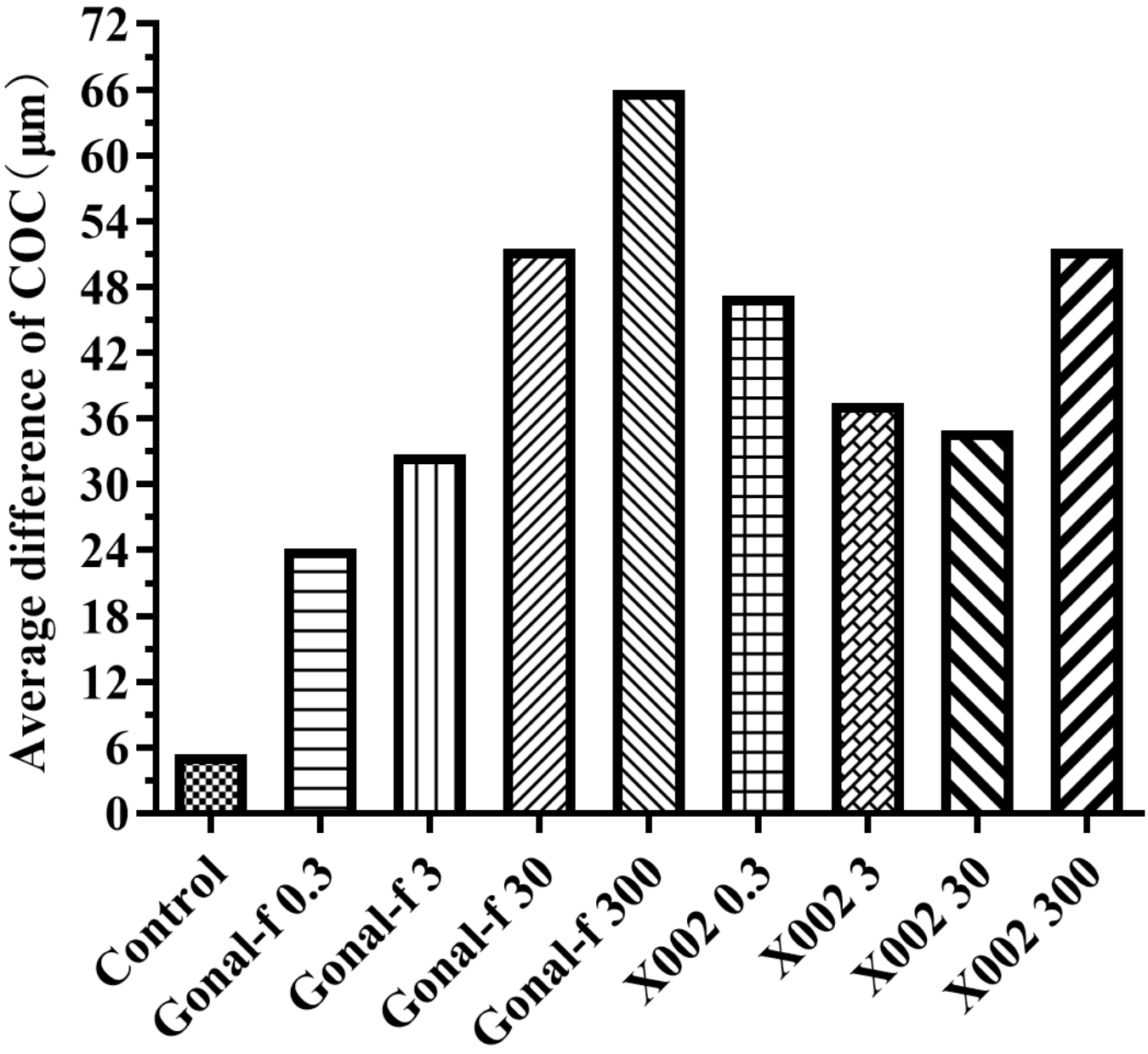

Figure 5

Photograph of COC after treatment with Gonal-f or X002 at different concentrations. COCs (n = 80–100 per group) were treated with Gonal-f or X002 (0.3, 3.0, 30.0, or 300.0 nmol/L) for 14 h. At 0h and 14h, COCs were photographed.

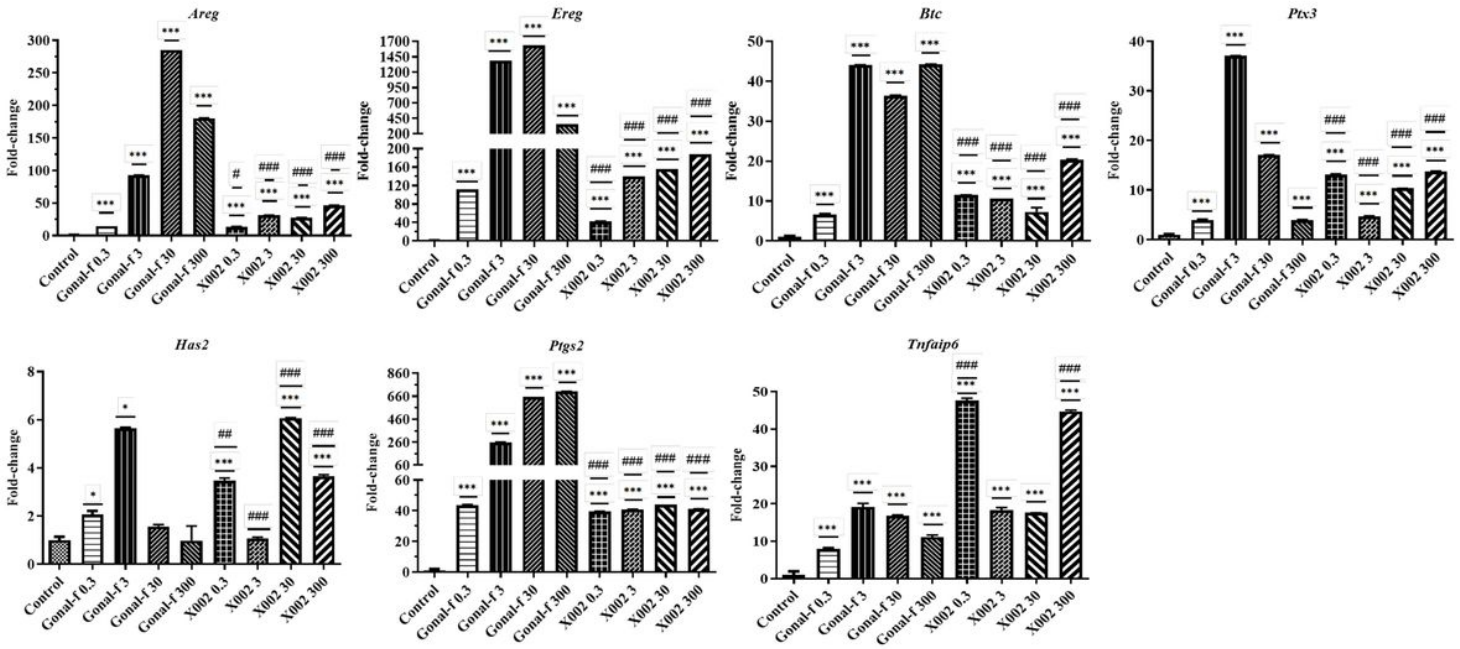

Figure 6

Expression levels of genes related to COC expansion after treatment with Gonal-f or X002 at different concentrations. Expression of genes involved in COC expansion determined by RT-qPCR. Each gene was normalized to endogenous β-actin mRNA levels and the fold change was determined by setting the relative transcript level of the control sample to 1 [6]. COCs (n = 80–100 per group) were treated with Gonal-f (0.3 nmol/L) or X002 (0.3, 3.0, 30.0, or 300.0 nmol/L) for 14 h. One-way analysis of variance method was used to compare between Control, Gonal-f and X002. Compared to Control, *P < 0.05, **P < 0.01, ***P < 0.001, no significant difference was noted between Control and Gonal-f or X002. Compared to Gonal-f at the same concentration, #P < 0.05, ##P < 0.01, ###P < 0.001, no significant difference was noted between Gonal-f and X002 at the same concentration.

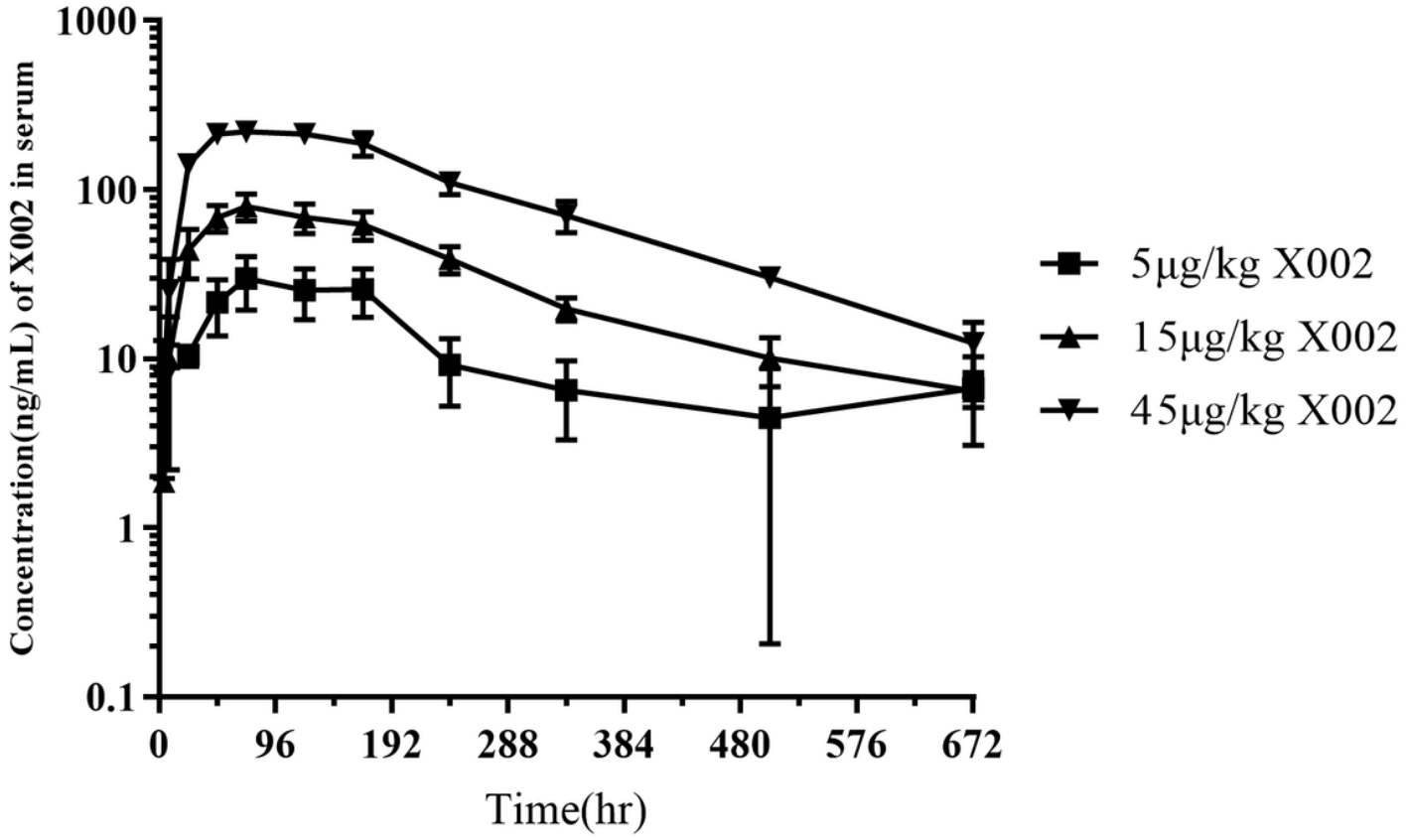

Figure 7

Pharmacokinetics of X002 in rats. After a single subcutaneous injection of X002(5, 15, 45 μg/kg), blood samples were collected at different time points. Serum X002 concentrations were tested by ELISA on plates coated with FSH antibody.

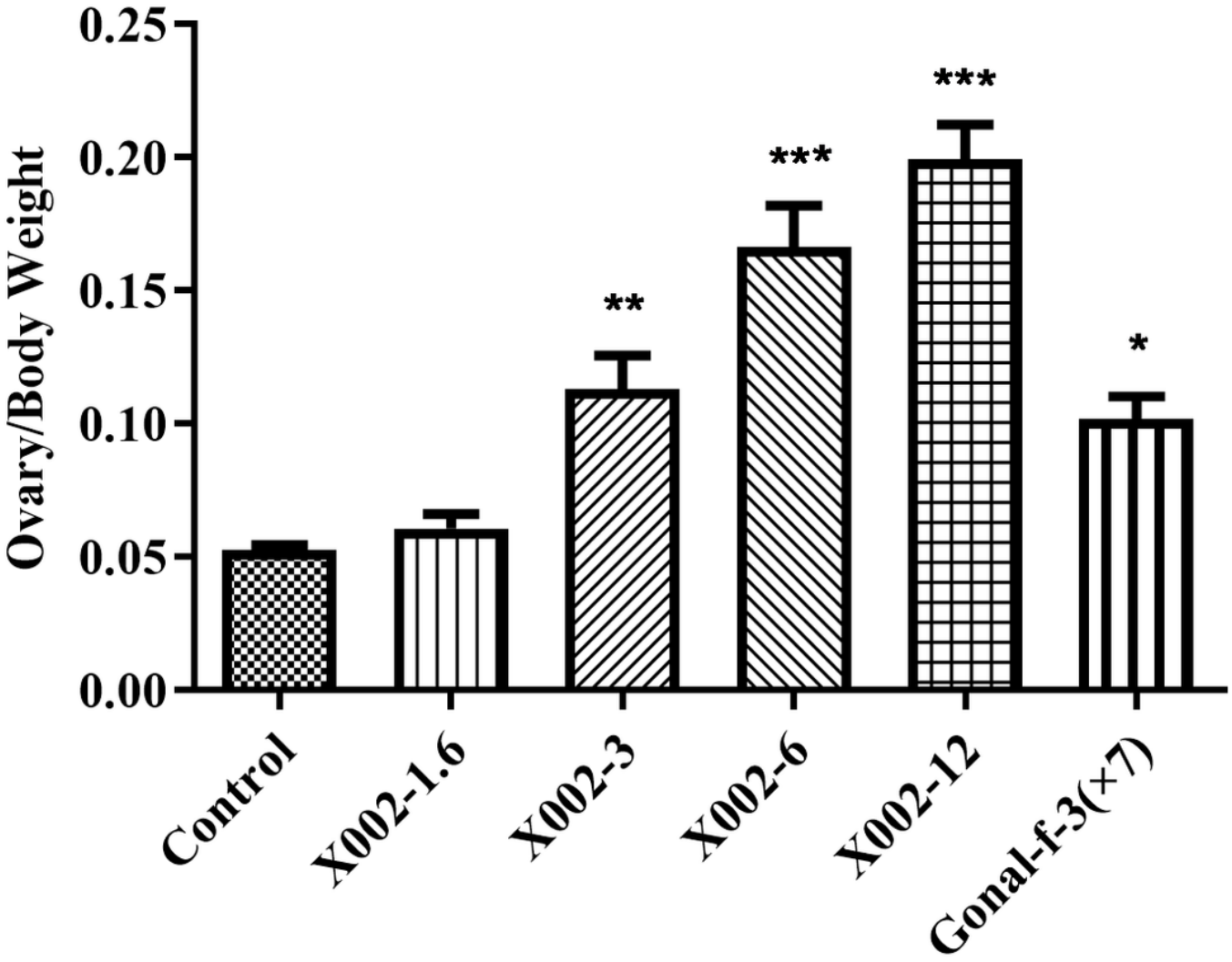

Figure 8

Rats were subcutaneous injected with Gonal-f (7 injections, with an interval of 12 h between each injection, 3 pmol) or X002(single injection, 1.6\3\6\12 pmol). 84h after X002 treatment, rats were injected with 3.75 IU hCG. Then, 12 h after hCG treatment，ovary and body weight were weighted. One-way analysis of variance method was used to compare between Control, Gonal-f and X002. Compared to Control, *P < 0.05, **P < 0.01, ***P < 0.001. No significant difference was noted between Gonal-f and X002 at the same concentration.

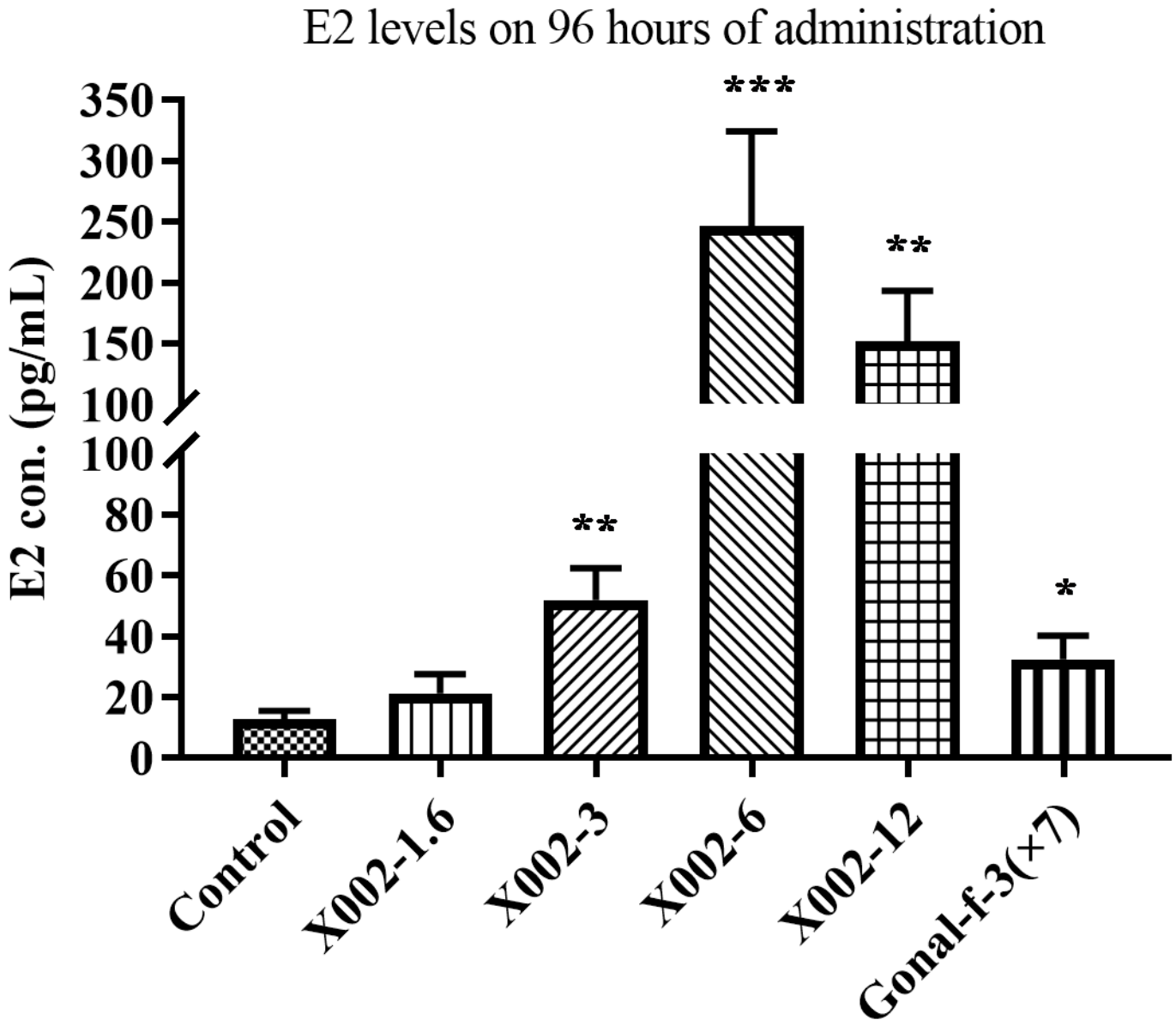

Figure 9

Serum E2 level in rats at 96 h. Rats were subcutaneous injected with Gonal-f (7 injections, with an interval of 12 h between each injection, 3 pmol) or X002(single injection, 1.6\3\6\12 pmol). 84h after X002 treatment, rats were injected with 3.75 IU hCG. Then, 12 h after hCG treatment, blood samples were collected. Serum E2 concentrations were tested by rat E2 ELISA kit. One-way analysis of variance method was used to compare between Control, Gonal-f and X002. Compared to Control, *P < 0.05, **P < 0.01, ***P < 0.001. No significant difference was noted between Gonal-f and X002 at the same concentration.

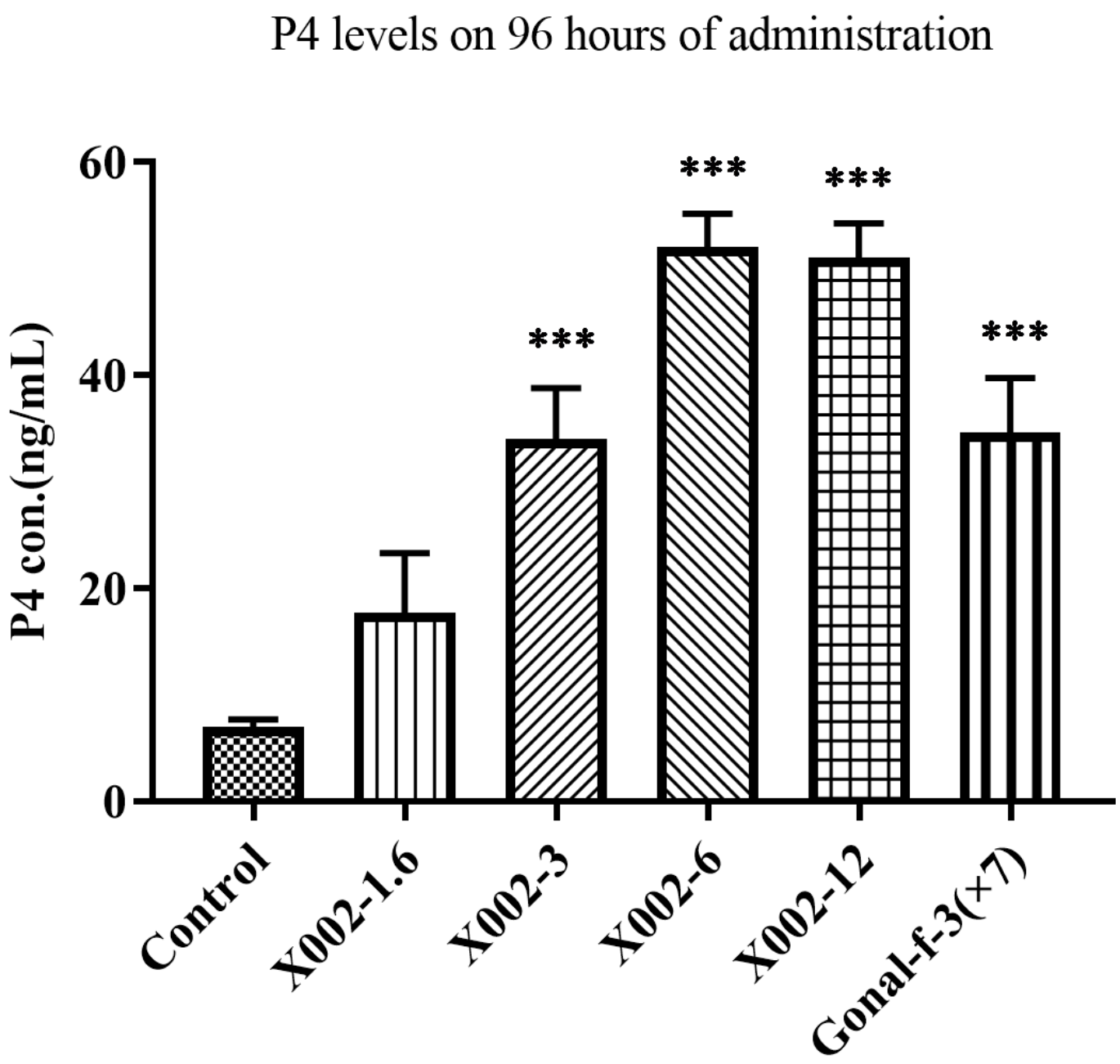

Figure 10

Serum P4 level in rats at 96 h. Rats were subcutaneous injected with Gonal-f (7 injections, with an interval of 12 h between each injection, 3 pmol) or X002(single injection, 1.6\3\6\12 pmol). 84h after X002 treatment, rats were injected with 3.75 IU hCG. Then, 12 h after hCG treatment, blood samples were collected. Serum P4 concentrations were tested by rat P4 ELISA kit. One-way analysis of variance method was used to compare between Control, Gonal-f and X002. Compared to Control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Compared to Gonal-f at same dose, there was no significant difference.

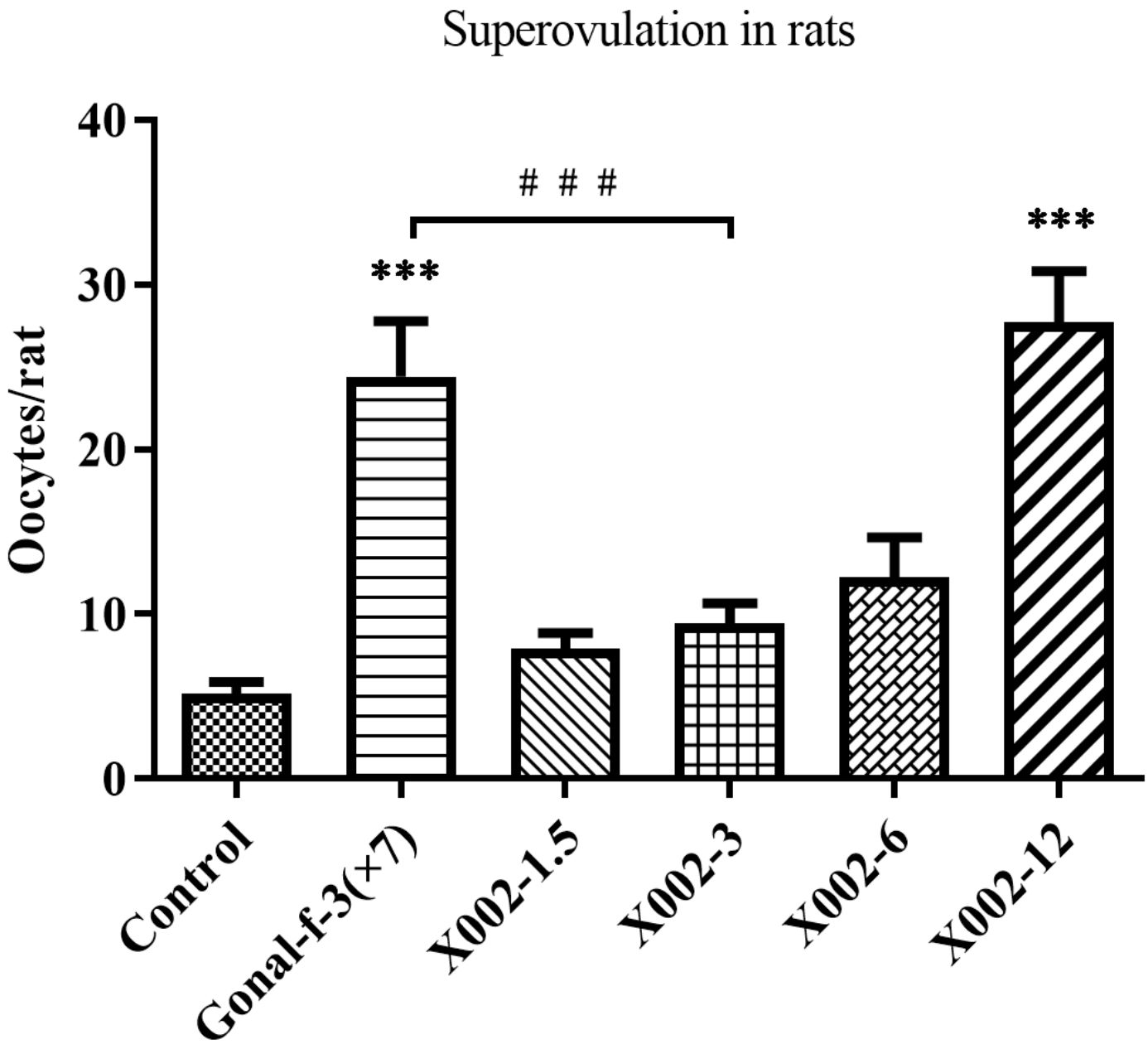

Figure 11

Number of ovulated oocytes in rats at 108 h. Superovulation was administered by Gonal-f (7 injections in total, with an interval of 12 h between two injections) and X002 (single injection, 1.5\3\6\12 pmol). Rats were induced by 20 IU of hCG at 84 h after the first administration, then 24 h after hCG injection, numbers of oocytes were counted. One-way analysis of variance method was used to compare between Control, Gonal-f and X002. Compared to Control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Compared to Gonal-f at same dose, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.