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Research

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# Comparative superovulation outcomes across estrous phases using PMSG-hCG and rFSH-rhCG in mice

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## Abstract

**Background** Enhancing stimulation efficiency in mice supports the application of assisted reproductive technologies (ART) for species conservation.

**Objective** This study compared the stimulatory potential of two ART-supporting hormone protocols: the conventional PMSG-hCG combination and recombinant gonadotropins (rFSH-rhCG), a newer generation produced by genetic engineering.

**Methods** Sixty-three female mice from four distinct estrous phases were used in this study. Both hormone regimens, with FSH- and LH-like activities, were administered sequentially between 4:00 and 5:00 pm, at a 47–48 h interval. Stimulation success was evaluated based on the proportion of females showing a positive response and the number of oocytes retrieved.

**Results** The diestrus phase yielded the highest response for both treatments. Approximately 60% of the females responded to PMSG-hCG, and 80% responded to recombinant hormones. The number of oocytes recovered reached 239 in the PMSG-hCG group and 137 in the recombinant group. Interestingly, recombinant hormone administration during estrus induced ovulation of 400 oocytes; however, the efficiency ratio was lower than that during diestrus (30.8 vs 34.3).

**Conclusion** Both PMSG-hCG and recombinant gonadotropins effectively stimulate ovulation. The diestrus phase provides the most consistent results; therefore, diestrus is recommended as the optimal stage for superovulation protocols in mice.

**Keywords** estrus cycle, follicle stimulation, mice, ovulation, recombinant gonadotropin hormone.

## Introduction

High-quality oocytes are essential for successful fertilization and embryo development during in vitro reproductive procedures. These oocytes are crucial not only for basic reproductive studies and assisted reproductive techniques but also for applications in genetic resource conservation and endangered species protection (Jewgenow & Zahmel, 2020), transgenesis (Delerue & Ittner, 2017), or somatic nuclear transfer/

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cloning (Plautz *et al.*, 2016). In addition to reproductive purposes, oocytes are used to derive embryonic stem cells, which have potential applications in regenerative medicine.

In mice, 8-10 oocytes are ovulated, and nearly all oocytes are fertilized via natural mating (Nakao *et al.*, 2023). These numbers can be increased through superovulation. This stimulation technique employs a pair of exogenous gonadotropins to induce the development and ovulation of multiple oocytes. Usually, one hormone induces follicle development, oocyte maturation, and estrogen secretion, whereas the second triggers ovulation. Follicular development-inducing hormones are follicle-stimulating hormone (FSH)-like, such as pregnant mare's serum gonadotropin (PMSG) and human menopausal gonadotropin (hMG), whereas ovulation induction uses luteinizing hormone (LH)-like hormones, such as human chorionic gonadotropin (hCG). This technique has been used to obtain millions of embryos from hundreds of mouse strains (Whiting, 2018).

Recent advances in biotechnology have facilitated the development of recombinant hormones. Recombinant biological products are proteins produced in vitro using recombinant DNA technology, which uses biological processes to produce large-molecule drugs without synthetic chemistry. These hormones are produced in the ovarian cells of Chinese hamsters (CHO) (Lunenfeld *et al.* 2019; Abdallah *et al.*, 2023). Compared to hormones extracted from human biological fluids, recombinant gonadotropins offer better purity, higher specific activity, batch-to-batch consistency, and reduced risk of contamination. These recombinant hormones are used in both assisted reproductive technology (ART) and the treatment of reproductive disorders.

In vertebrates, natural gonadotropins regulate the development and function of the gonads and testes in males and ovaries in females. Human gonadotropins include FSH, LH, and hCG. Recombinant FSH (rFSH) is a safe, effective, and widely used substance for the treatment of fertility disorders. Recombinant LH (rLH) is also recommended as an adjunct to rFSH for ovulation induction in hypogonadotropic women and is commonly used for multiple follicular development in infertile ovulatory women who undergo ART procedures, such as in *in-vitro* fertilization (IVF) and embryo transfer (Conforti *et al.* 2021; Alviggi *et al.*, 2025).

In the superovulation technique, recombinant hormones are functionally comparable to the commonly used stimulating hormone (PMSG-hCG). However, it remains unclear whether the recombinant hormone (rFSH-rhCG) is comparable to PMSG-hCG stimulation in terms of the number and quality of ovulated oocytes. Additionally, the effects of recombinant hormones on the estrus cycle and ovulation timing are not fully understood. The estrus cycle reflects dynamic changes in reproductive hormones and ovarian activity regulated by gonadotropins. Therefore, this study aimed to compare the effectiveness of conventional stimulation using PMSG-hCG and recombinant hormonal stimulation in mice. These findings are expected to provide insights into more effective stimulation protocols and contribute to the refinement of experimental reproductive techniques.

The success of hormonal stimulation can be assessed immediately after ovulation or post-fertilization (e.g., day 3 post-hCG). While immediate evaluation enables direct assessment of oocyte number and quality, it is constrained by the rapid decline in oocyte viability after six hours post-ovulation (Abdallah *et al.*, 2023). In this study, stimulation success was evaluated post-fertilization by embryo collection, allowing the confirmation of ovulation, fertilization capacity, and early embryonic development (Roque & Sunkara 2025). However, this method requires the use of fertile males to minimize variability in sperm quality.

# Methods

### Ethical statement

This study was conducted at the Embryology Laboratory of the School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, Indonesia. It is part of the research project titled *"Reconstruction of parthenogenetic and fertilized mouse embryos as a model for early embryonic development"* and was approved by the Animal Ethics Commission of the School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, Indonesia (No. 122/KEH/SKE/IV/2019).

### Research design

A total of 63 female mice and 16 male mice (*Mus musculus*) were used in this study. Prior to treatment, all animals underwent a two-week acclimatization period to allow adaptation to laboratory conditions, including temperature, humidity, light cycle, and handling. During this period, mice were provided ad libitum feed and drinking water and were treated with topical antiparasitic (deltamethrin 0.6%).

This study employed a two-group experimental design to compare the efficacy of conventional stimulation using PMSG-hCG (Folligon-Chorulon; control group) with a relatively new stimulation protocol employing recombinant gonadotropins (rFSH-rhCG; Pergoveris®-Ovidrel®). Female mice were randomly assigned to two groups: the conventional group (n = 31) received PMSG-hCG, while the recombinant group (n = 32) received rFSH-rhCG. Both PMSG and rFSH were administered between 4:00 and 5:00 pm, followed by hCG or rhCG injections 47–48 hours later. An overview of this procedure is presented in **Figure 1**.

In contrast, all male mice were untreated and served solely for breeding purposes. Each male was paired with one female from each experimental group to ensure natural fertilization following a 1:1 male-to-female mating ratio.

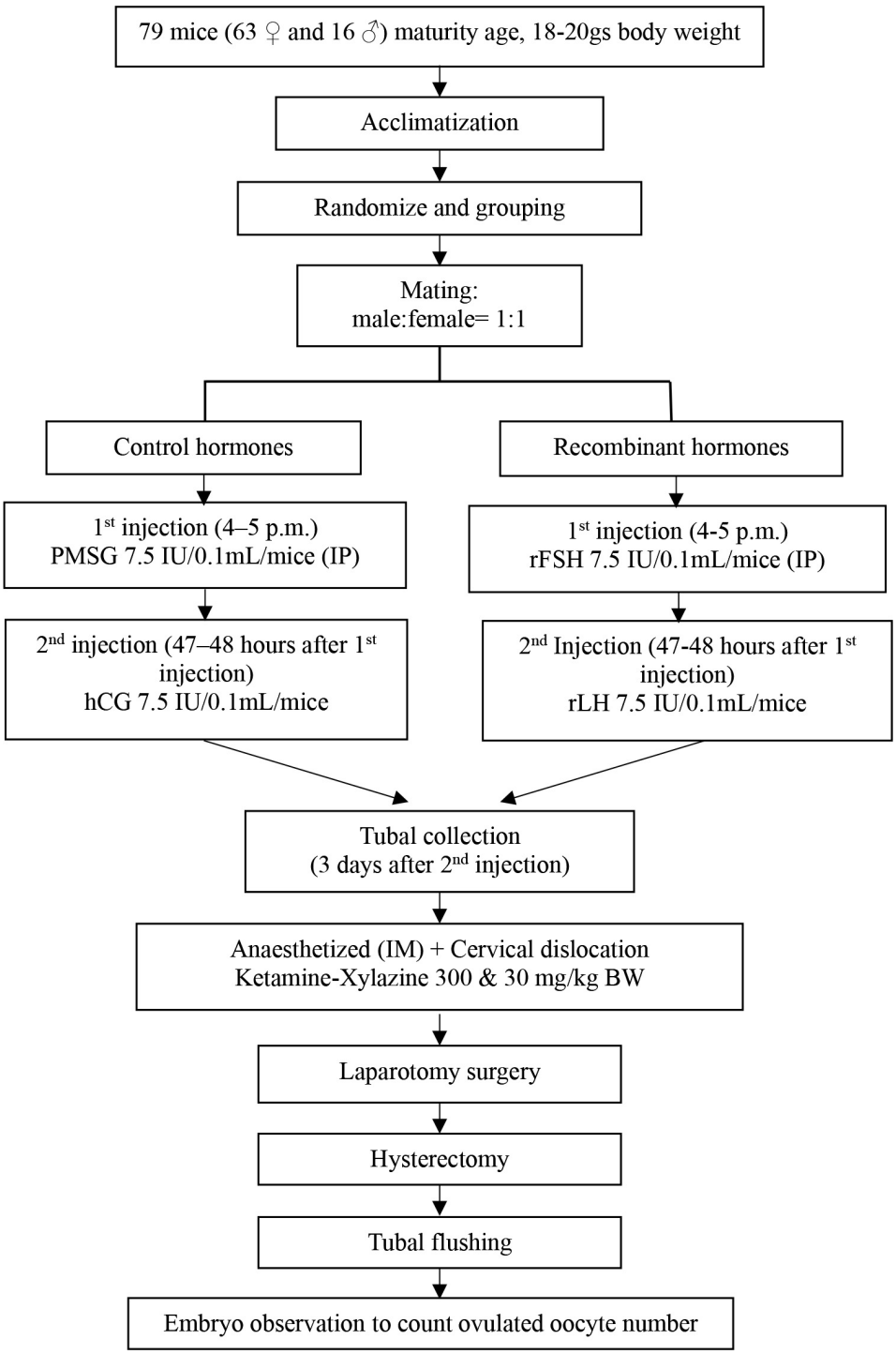


**Figure 1** Design experiment and superovulation protocol using conventional (PMSG-hCG) and recombinant (rFSH-rhCG) hormones accros estrous phases in mice

### Animal preparation

The mice were housed in standard plastic cages (27 cm × 22 cm × 9 cm) during the acclimatization period. Female mice were randomly grouped with approximately five animals per cage, whereas males were housed individually. All animals were sexually mature and aged six weeks or older. Following acclimatization, the females were divided into two groups according to the research design. The animals were provided ad libitum feed and drinking water, as well as routine feeding and cage maintenance throughout the study.

### Vaginal smear

Vaginal smears were performed using the vaginal flushing technique to monitor the estrus cycle prior to hormonal stimulation. Vaginal fluid was collected daily between 3:00 and 5:00 pm using a blunt-modified plastic syringe filled with 0.9% NaCl. The saline was gently flushed into the vaginal canal and aspirated to collect the superficial epithelial cells. The fluid was then smeared onto glass slides and air-dried. Fixation was performed with 70% methanol for 5 min, followed by Giemsa staining for approximately 25 min. Excess stain was

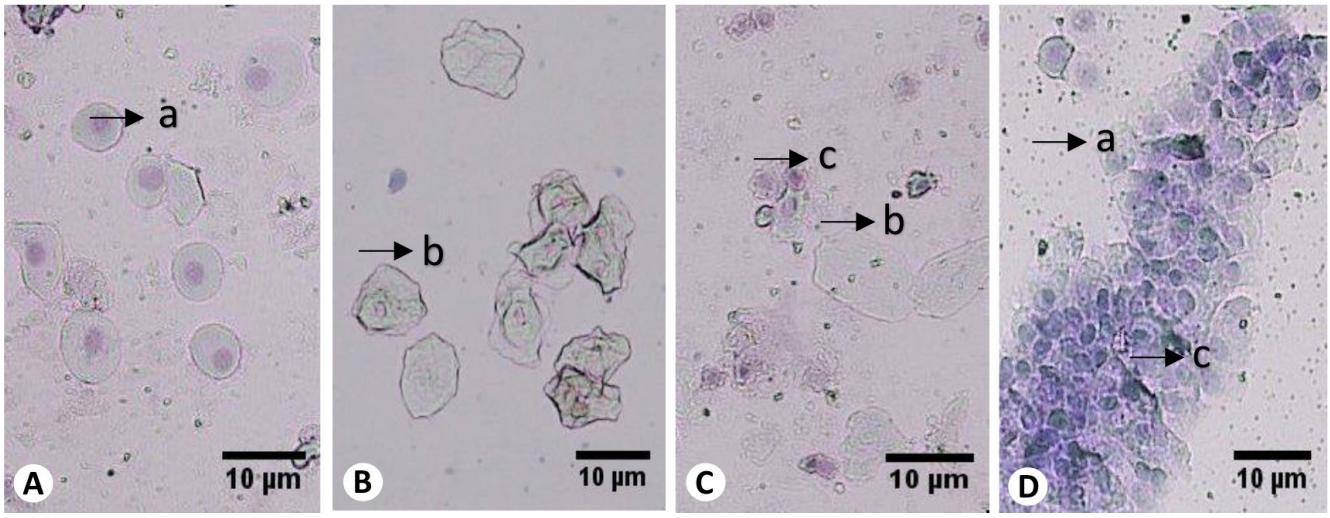


**Figure 2** The vaginal smear of four phases of the estrus cycle in female mice: A. Proestrus phase with nucleated epithelium cells dominantly (a), B. Estrus phase with nucleated epithelium cells found has changed into cornified epithelium cells (b), C. Metestrus phase with cornified epithelium cells and leukocytes (c), D. Diestrus phase with some nucleated epithelium cells and leukocytes present.

removed by rinsing with tap water, and the slides were air-dried prior to microscopic examination.

Samples were observed under a light microscope at 40× magnification to determine the stages of the estrus cycle. The identified cell types included round nucleated epithelial cells, non-nucleated cornified epithelial cells, and leukocytes. The relative proportions of these cells were used to classify estrous stages as proestrus, estrus, metestrus, or diestrus.

### Superovulation and embryo collection to count ovulated oocytes number

Female mice were superovulated via intraperitoneal injection of 7.5 IU PMSG, followed 47–48 h later by 7.5 IU hCG as the control/conventional stimulation group. The recombinant group received equivalent doses of rFSH and rhCG (**Figure 1**). Immediately after LH-like hormone injection (hCG and rhCG), females were paired with males in a 1:1 ratio for natural mating.

Embryos were collected three days after LH-like hormone administration. Female mice were euthanized for embryo retrieval using a combination of anesthesia and cervical dislocation. Anesthesia was administered intramuscularly using ketamine (300 mg/kg BW) and xylazine (30 mg/kg BW). Cervical dislocation was performed only after the animals had been fully anesthetized. The reproductive tract, including the ovaries, oviduct, and uterus, was excised and placed in GMOPS medium supplemented with 1% fetal bovine serum (FBS) at 37°C. The embryos were retrieved by flushing the oviductal ampulla with the same medium. The procedure was performed under a stereomicroscope at 100× magnification. The total number of embryos collected (both normal and abnormal/degenerated) was used as an indicator of ovulated oocytes.

### Data analysis

Statistical analysis was performed using the Minitab 20 software. Stimulation response data were analyzed using the Mann-Whitney U test, while the number of oocytes was analyzed using the independent Student's *t*-test.

## Results

Estrus cycles were identified through morphological changes in the ovaries, uterus, and vagina as well as through cytological analysis of vaginal smears. Vaginal flushing revealed distinct cellular profiles corresponding to four estrus phases: proestrus, estrus, metestrus, and diestrus. Each phase was characterized by the predominance of specific epithelial cell types. Proestrus was marked by the presence of round nucleated epithelial cells (**Figure 2A**). The estrus phase showed abundant cornified epithelial cells without leukocytes (**Figure 2B**). The metestrus phase was characterized by a thinner epithelial layer with the initial appearance of leukocytes (**Figure 2C**). In the diestrus phase, leukocytes dominated the smear and were accompanied by occasional nucleated epithelial cells (**Figure 2D**).

The stage of the estrus cycle influences the outcome of superovulation. Although all phases responded to hormonal stimulation, the number of ovulated oocytes varied across estrus cycle stages and treatment groups. **Table 1** presents the responsiveness of the mice to PMSG-hCG and recombinant hormone administration.

Overall, the diestrus phase yielded the highest superovulation response, both in terms of the number of oocytes collected and the proportion of responsive individuals. Mice stimulated during this phase produced 239 oocytes after PMSG-hCG administration and 137 oocytes after recombinant hormone administration

**Table 1** Number of mice accros estrous phases responding to the stimulation using conventional (PMSG-hCG) and recombinant (rFSH-rhCG) hormones

| Hormone treatment | Superovulation responsiveness level (%) | | |
|---|---|---|---|
| | N | + | - |
| Proestrus | | | |
| PMSG-hCG | 4 | 1 (25) | 3 (75) |
| rFSH-rhCG | 4 | 1 (25) | 3 (75) |
| Estrus | | | |
| PMSG-hCG | 10 | 4 (40) | 6 (60) |
| rFSH-rhCG | 20 | 13 (65) | 7 (35) |
| Metestrus | | | |
| PMSG-hCG | 5 | 1 (20) | 4 (80) |
| rFSH-rhCG | 3 | 1 (33) | 2 (67) |
| Diestrus | | | |
| PMSG-hCG | 10 | 6 (60) | 4 (40) |
| rFSH-rhCG | 5 | 4 (80) | 1 (20) |

The (+) sign indicates successful stimulation with ≥10 ovulated oocytes, and the (–) sign indicates the contrary condition with the number of ovulated oocytes. The numbers in parentheses indicate the percentage of mice that were responsive to the same treatment. The data in each phase were not significantly different ($P > 0.05$).

(**Figure 3D**). This finding indicates a higher ovulatory potential during the diestrus phase.

Interestingly, recombinant hormone stimulation during the estrus phase resulted in the highest absolute number of oocytes collected (400 oocytes) (**Figure 3B**). However, the efficacy ratio (total oocytes/number of mice showing a positive stimulation response) was lower than that during the diestrus phase (30.8:34.3). The lowest oocytes yield in the recombinant group occurred during proestrus (**Figure 3A**), while it was the lowest in the conventional group (PMSG-hCG) noted during metestrus (only 47 oocytes were retrieved, **Figure 3C**).

# Discussion

The estrous cycle is a key indicator of ovarian activity. The proliferation and differentiation of epithelial cells in the reproductive tract are primarily regulated by estrogen and progesterone. Roque and Sunkara (2025) reported that estrogen levels are relatively low during the proestrus phase but increase during estrus. In the present study, vaginal smears revealed a predominance of nucleated epithelial cells during the proestrus phase (**Figure 2A**). This is consistent with Aritonang *et al*. (2017), who noted that vaginal smears during this phase typically contain numerous nucleated epithelial cells and occasional leukocytes. Serum estradiol levels peak during proestrus and are accompanied by elevated FSH concentrations.

Increased estrogen levels stimulate the release of gonadotropin-releasing hormone (GnRH), which in turn promotes the secretion of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Estrogen also thickens the vaginal epithelium, leading to rapid cornification. The estrus phase was marked by the abundance of cornified epithelial cells and the absence of leukocytes (**Figure 2B**). Estradiol promotes stratification and cornification of the vaginal mucosa; conversely, estradiol withdrawal induces epithelial desquamation. Interestingly, estradiol concentrations during estrus are significantly lower than those during proestrus, but rise again in metestrus.

Hormone administration in mice during diestrus yielded the highest stimulation response to the conventional stimulation hormone (PMSG-hCG). A natural increase in endogenous FSH characterizes the diestrus phase and is in the luteal phase of a follicular wave without a dominant follicle (Liamanu *et al.*, 2018). According to Setiadi *et al.* (2005), gonadotropin administration in the absence of a dominant follicle enhanced superovulation. Thus, exogenous FSH (either PMSG or rFSH) may act synergistically with endogenous FSH, resulting in significantly increased ovulation rates during diestrus.

In contrast, stimulation during proestrus produced a lower number of ovulations (marked by fewer collected oocytes; **Figure 3B**). Although FSH levels are the highest during this phase (Aritonang *et al.*, 2017), the presence of Graafian follicles on the surface of the ovary secretes inhibin, which exerts FSH-negative feedback, suppresses the growth of subordinate follicles, and often results in follicular atresia (Woldemeskel, 2017). Consequently, both conventional and recombinant treatments showed low stimulation efficacy, fewer mice with positive stimulation responses, and fewer oocytes retrieved.

Interestingly, recombinant hormone administration during estrus elicited a strong stimulatory response. This may be attributed to the prolonged half-life of the recombinant hormones. Cetinkaya *et al*. (2025) reported a half-life of 5 h for subcutaneous and 8 h for intramuscular recombinant FSH administration, with a terminal half-life of approximately 37 h. Given that the estrus phase lasts 12–48 h (Ajayi & Akhigbe, 2020), exogenous hormone activity may extend into the subsequent metestrus phase, supporting continued follicular development and ovulation. This sustained action may explain the high number of ovulated oocytes during the estrus phase stimulation despite the inhibitory presence of dominant follicles.

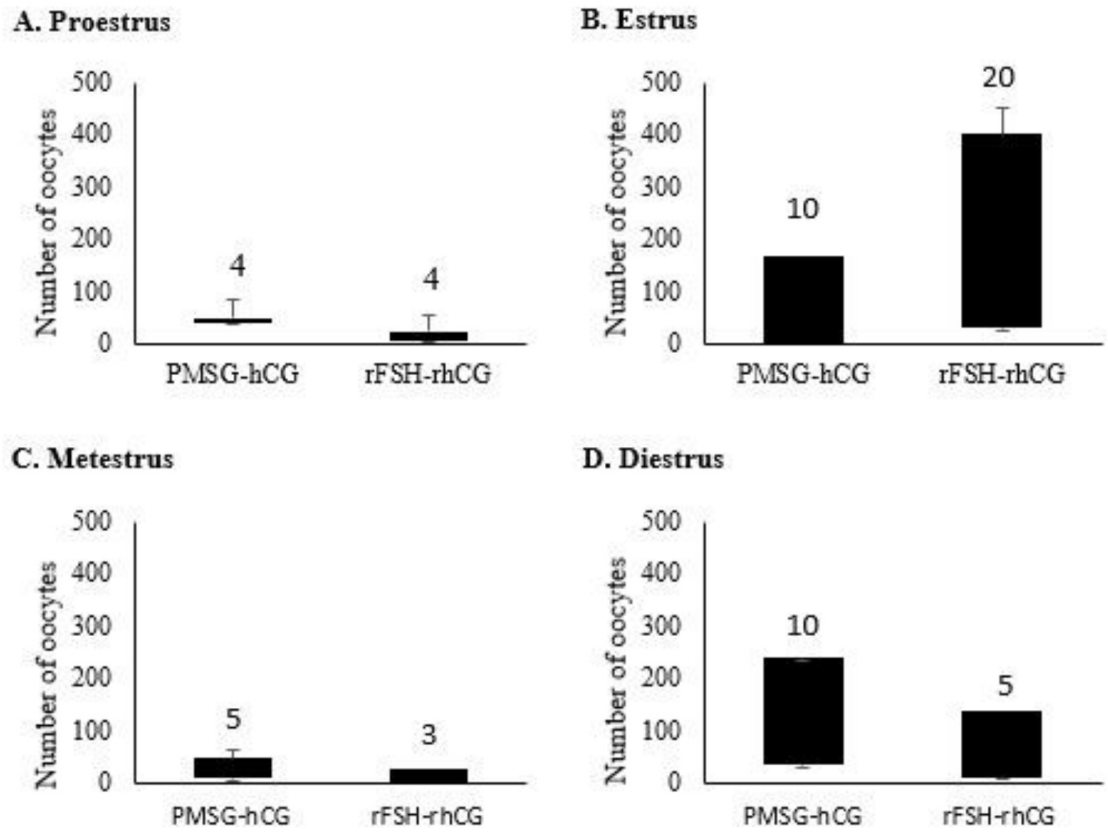


**Figure 3** Number of ovulated oocytes in estrus phases stimulated by common superovulation hormone (PMSG-hCG) and recombinant hormone treatments. The data in each phase have no significant difference ($p>0.05$). The number in the column shows the number of mice (n).

Following ovulation, new follicular growth begins during estrus. However, endogenous FSH levels are the lowest during this phase (Aritonang *et al*., 2017). Exogenous hormones may support early follicle development, but large-cohort stimulation can lead to follicular atresia if hormone levels are insufficient to sustain growth through ovulation. Ajayi and Akhigbe (2020) reported a high number of early-stage and dormant follicles in mice during this phase, but hormone administration may be insufficient to induce ovulatory maturity in these follicles. Therefore, only a small number of mice showed a positive response after the hormone injection.

In line with previous studies (Nakao *et al.*, 2023; Abdallah *et al.*, 2023), this study confirmed that superovulation protocols (both PMSG-hCG and recombinant hormones) significantly increased the number of oocytes collected compared with natural ovulation (usually 8–12 oocytes). The highest ovulation was obtained when stimulation occurred during diestrus (**Figure 3D**), when numerous secondary and preantral follicles were present, and no dominant follicles or inhibin secretion occurred. The synergy between exogenous and endogenous hormones during this phase allows optimal follicular development. Prolactin, secreted by the anterior pituitary during diestrus, prolongs this phase and supports follicular growth in the absence of inhibin (Szukiewicz, 2024). In addition, LH-like compounds (such as hCG) play an essential role in oocyte maturation. Once hCG reaches the ovary, it mimics LH to induce meiotic resumption and ovulation (Arroyo *et al*., 2020). Therefore, the diestrus phase provides an optimal hormonal environment for superovulation.

The lowest ovulation number following recombinant treatment was observed in the proestrus phase (**Figure 3A**), in which the presence of dominant follicles and high inhibin levels suppressed the growth of subordinate follicles. Excess estrogen may also suppress FSH secretion, further limiting follicular recruitment (Satué & Gardon, 2020). Receptor inactivity may be another limiting factor. For example, progesterone has been shown to inactivate receptors by promoting degradation or preventing hormone binding (Ye *et al.,* 2017). Hormonal stimulation during this phase resulted in the lowest ovulation response.

In the estrus phase, recombinant hormone treatment yielded a high number of ovulations (**Figure 3B**). Dominant follicles respond to LH-like stimuli, whereas subordinate follicles are suppressed. This suggests that not all Graafian follicles were ovulated by endogenous LH, and that exogenous hormones may have recruited additional follicles. Another possible explanation is that FSH-like compounds promoted early folliculogenesis and enhanced the transition from the secondary to Graafian

stages. The long half-life of recombinant hormones allows prolonged stimulation, possibly overlapping with the metestrus phase, thereby contributing to continued follicular maturation.

Despite the high oocyte yield in the estrus-phase recombinant treatment, its efficacy was slightly lower than that in diestrus (30.8 vs. 34.3). Of the 20 mice stimulated during estrus, 13 responded positively, contributing to a high total oocyte count.

Unlike the recombinant treatment, PMSG-hCG administration during metestrus resulted in the lowest collected oocyte count (**Figure 3C**). This may be due to suboptimal timing, where follicular development was initiated but not sustained because of declining hormone levels. LH-induced leukocyte infiltration is essential for ovulation and corpus luteum formation. Aritonang *et al*. (2017) observed a leukocyte surge after hCG injection several hours post-injection, which was likely too late to rescue follicular development initiated during metestrus. Consequently, ovulatory success in this phase is markedly lower.

Overall, stimulation in mice resulted in favorable superovulatory outcomes with both conventional stimulation using PMSG-hCG and recombinant hormones (rFSH-rhCG) during the diestrus phase. However, extrapolation of these findings to other species requires further investigation, as physiological differences may exist, such as variations in the duration and pattern of the estrous cycle, species-specific hormonal responses, and differences in hormonal metabolism.

## Conclusion

Conventional (PMSG-hCG) and recombinant stimulatory hormone administration produced the most effective superovulatory response during the diestrus phase. Therefore, hormonal stimulation during diestrus using either conventional or recombinant gonadotropins is recommended for optimal outcomes. Further research is warranted to investigate the pharmacokinetics, particularly the extended half-life of recombinant hormones, following intraperitoneal administration in mice.

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**Conflict of interest** The authors declare no conflicts of interest regarding the publication of this paper.

**Author contribution** YNM and CBS: Formal analysis, investigation, project administration, software, visualization, writing – original draft, DNP and NMDH: Conceptualization, data curation, methodology, resources, supervision, validation, writing – review & editing, AB: Conceptualization, data curation, funding acquisition, methodology, resources, supervision, validation, writing – review & editing.

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