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Original Article

The effect of fixed-time artificial insemination protocol initiated at different stages of the estrous cycle on follicle development and ovulation in gilts

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Abstract. Hormonal products have been developed for fixed-time artificial insemination (FTAI) to improve the efficiency of swine production. Here, we evaluated the effect of an FTAI protocol initiated during different phases of the estrous cycle on follicle development and ovulation in gilts. A total of 36 gilts were equally divided into three groups designated as the luteal (L), follicular (F), and post-ovulation (O) groups and fed with 20 mg of altrenogest for 18 days, followed by intramuscular injection of 1000 IU PMSG at 42 h after withdrawal of altrenogest, and 100 μg of GnRH after an 80-h interval. The L group had the highest number of follicles 4–6 mm in diameter, as well as corpora hemorrhagica. The mRNA expression of caspase-9 in the L group were significantly lower than those in the O and F groups ($P < 0.05$), while CYP11A1 and VEGF mRNA expression levels were significantly higher ($P < 0.05$). Moreover, FSHR mRNA levels were significantly higher in the O group than in the L, F, and control groups ($P < 0.05$). LHCGR and CYP19A1 mRNA levels were the highest in the F group ($P < 0.05$). Thus, the changes in the expression of genes associated with follicular development, maturation, and ovulation identified in this study indicated that initiation of the FTAI protocol during the luteal phase induced a better environment for follicle development and ovulation in gilts.
Key words: Altrenogest synchronization, Estrous cycle, Fixed time artificial insemination (FTAI), Gene expression
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The growth, development, maturation, and ovulation of ovarian follicles are complex physiological processes that are regulated by endocrine hormones, the immune system, metabolic signals, and other factors, such as intrafollicular paracrine factors from the theca, mural, and cumulus granulosa cells, as well as the oocyte itself [1–3]. In this intricate regulatory network, endocrine hormones, including progesterone (P4), follicle-stimulating hormone (FSH), luteinizing hormone (LH), gonadotropin-releasing hormone (GnRH), and estradiol (E2), play important roles in the different stages of follicular development during the estrous cycle [4–6]. Therefore, a variety of hormone products and analogs, such as altrenogest, pregnant mare serum gonadotropin (PMSG), equine chorion gonadotropin (eCG), GnRH, D-Phe6–LHRH (a GnRH agonist), and triptorelin (a GnRH agonist), have been developed to synchronize the estrous cycle and ovulation in domestic animals [7–11]. The use of these hormone products has led to the development of a novel technique, known as fixed-time artificial insemination (FTAI), which has been widely accepted in recent years by most large pig farms in China and several other countries [12, 13].

Currently, batch management systems are widely applied in most commercial swine farms and these systems have significantly improved production efficiency leading to economic benefits [14]. Employing the FTAI technique for gilts and sows can help farms achieve optimal batch management of swine [13]. In swine, the duration of a complete estrous cycle is approximately 19–21 days, where a cycle is defined as starting with the observation of behavioral estrus and ending with the next observation of behavioral estrus [15, 16]. In this estrous cycle, the development of ovarian follicles goes through different physiological stages: a luteal phase, a follicular phase, and an ovulation period [17]. In weaned sows, since follicle development and estrus synchrony are observed after weaning, FTAI protocols can be initiated at weaning or at a certain time after weaning [18–20]. Effective FTAI protocols are also available for cyclic gilts [12]. Moreover, some successful protocols employ altrenogest to avoid or reduce follicle growth and suppress ovulation in gilts. Martinat-Botte *et al.* observed estrus in 93% of altrenogest-treated gilts from 5 to 7 days after the withdrawal of altrenogest [21]. They also designed different protocols based on altrenogest, eCG, and GnRH agonists to synchronize estrus in cyclic gilts and observed an estrus rate of up to 100% using these protocols [22]. However, in FTAI protocols used for gilts, most treatments are initiated irrespective of the stage of the cycle [21, 23]. Although previous research successfully synchronized estrus and ovulation in gilts using exogenous hormones, few studies have conducted a detailed analysis of the effect of treatment initiation time based on the stage of follicular development.

Thus, the present study focused on evaluating the effect of an FTAI protocol initiated during different estrus phases on the ovarian follicles of gilts. In this study, the gilts were divided into three groups: a luteal phase group, a follicular phase group, and a post-ovulation group, according to the development of the ovarian follicles. We then examined follicular size and the number of corpora hemorrhagica. Moreover, we recovered early embryos and unfertilized oocytes and quantified the expression of genes associated with follicular growth, ovulation, and apoptosis.

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Materials and Methods

Animals and experimental design

This study was approved by the Animal Care and Use Committee of the Zhejiang Academy of Agriculture Science and performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals.

The experiments were performed at AnJi ZhengXin Animal Husbandry Co., Ltd., China. The gilts estrous was recorded each day according to the standing reflex and a total of 36 sexually mature gilts (Landrace × Yorkshire) were selected from a group of approximately 300 gilts with at least one estrus cycle, which were 220–240 days old and weighed approximately 120 kg. The 36 gilts were then equally divided into three groups according to the stage of their estrous cycle: a post-ovulation group (2–4 days after gilts displayed a standing reflex), a luteal group (10–12 days after gilts displayed a standing reflex), and a follicular group (17–19 days after gilts displayed a standing reflex).

In experiment 1, all animals were fed 20 mg of altrenogest (Ningbo Sansheng Biotechnology Co., Ltd., Ningbo, China) once a day for 18 consecutive days. Then, 1000 IU PMSG (Ningbo Sansheng Biotechnology Co., Ltd., Ningbo, China) was administered at 42 h after altrenogest withdrawal. Six animals in each group were slaughtered for sampling at 80 h post-PMSG administration. In experiment 2, the remaining gilts (n = 18) in each group received 100 μg of GnRH (Ningbo Sansheng Biotechnology Co., Ltd., Ningbo, China) via intramuscular injection at 80 h post-PMSG administration. AIs were performed at 24 and 40 h post-GnRH administration. Then, the animals were slaughtered for sampling at 5 h after the second AI (Fig. 1).

Follicular size measurement

In experiment 1, the ovaries were extracted at slaughter. The follicle and ovary diameters were determined using a ruler with 1 mm precision and were measured by averaging the diameter at the widest point and at a right angle to the diameter. The number of follicles in both ovaries with sizes of > 8 mm, 6–8 mm, 4–6 mm, and < 4 mm were recorded for each gilt.

Corpora hemorrhagica and embryo recovery

The remaining six gilts in each group included in experiment 2 were slaughtered at 5 h after the second AI and the reproductive organs were extracted at slaughter. The number of corpora hemorrhagica was estimated by counting the red bleeding points on the surface of the ovary. The presumed early embryos and unfertilized oocytes were recovered from the fallopian tubes and uterus using pre-warmed phosphate-buffered solution (PBS) supplemented with 2% fetal bovine serum.

Quantitative real-time PCR (RT-qPCR)

The follicular theca was collected from ovarian follicles with a size of 6–8 mm in the O, L, and F groups in experiment 1. Control follicles were collected from six randomly selected gilts that did not receive any treatment. Total RNA was extracted using an Animal Tissue Total RNA Kit (Hangzhou Xinjing Biological Reagent Development Co., Ltd., Hangzhou, China) and equal amounts of total RNA were reverse transcribed into cDNA using 20 μl of TransScript First-Strand cDNA Synthesis SuperMix (Beijing TransGen Biotechnology Co., Ltd., Beijing, China). RT-PCR was performed on a CFX96 Real-time PCR System (Bio-Rad Laboratories, Hercules, CA, USA) in a 10 μl reaction volume containing 0.5 μl of quantified cDNA, 5 μl of SYBR Green Realtime PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan), 0.5 μl of specific primers (10 μM) (Table 1), 1 μl of SYBR Green Realtime PCR Master Mix Plus, and 2.5 μl of distilled water. The cycling conditions were 95°C for 1 min and 40 cycles at 95°C for 15 sec, 57°C for 1 min, and 72°C for 15 sec. The 18s RNA gene was used as an internal control. Relative gene expression was semi-quantitatively analyzed using the $2^{-\Delta\Delta Ct}$ method ($2^{-\Delta\Delta Ct}$ = gene copy number in the test group/gene copy number in the control group). qPCR analysis was performed in triplicate for each sample.

Statistical analysis

Data are presented as the mean ± standard error. Statistical analyses were performed using SPSS Statistics for Windows (version 24.0; IBM SPSS Statistics, IBM Corp., Armonk, NY, USA). All data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Statistical significance was set at $P < 0.05$.

Results

The effects of the FTAI protocol on ovary size and the number of follicles

In experiment 1, the animals were slaughtered and their ovaries were collected before the administration of GnRH. As shown in Fig. 2, there was no significant difference in ovary size among the L (36 ± 3.46 cm, n = 6), O (33.83 ± 3.75 cm, n = 6), and F (34.92 ± 2.99

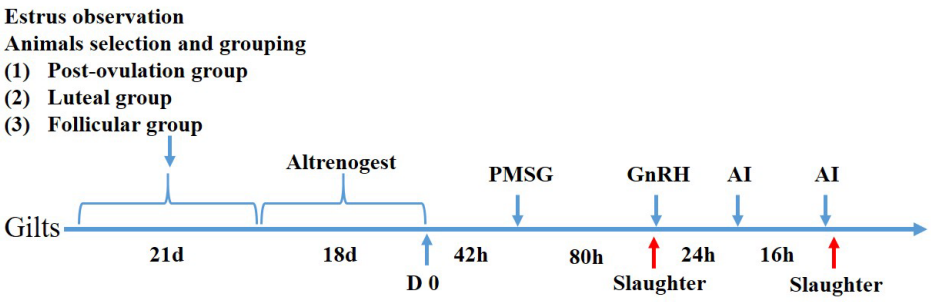

Fig. 1. Schematic representation of the experiment. Gilts were first divided into three groups, namely a luteal, follicular, and post-ovulation group. Animals were fed 20 mg of altrenogest for 18 days and then given an IM injection of 1000 IU PMSG at 42 h after withdrawal of altrenogest. At 80 h, 6 of the animals were slaughtered to examine ovary size and follicle size and to collect the parietal tissues of follicles with diameters of 6–8 mm. The remaining animals in each group were administrated 100 μg of GnRH at 80 h after PMSG injection. AIs were performed at 24 h and 40 h after GnRH administration. The animals are slaughtered 5 hours after the second AI to count the corpora hemorrhagica and to recover the embryos.

Table 1. List of primers for RT-qPCR

Target gene	Primers	PCR product size	Genbank accession number
Caspase-3	Forward: 5'-AGTCCCACTGTCCGTCTCAATC-3' Reverse: 5'-CTAACTGGCAAACCCAAACTTTTC-3'	84	NM_214131.1
Caspase-8	Forward: 5'-AGGCCCTGCTGAAGGAAAATCT-3' Reverse: 5'-CCTGTTCTCCCAGACAGTCC-3'	162	NM_00103177
Caspase-9	Forward: 5'-AACTTCTGCCATGAGTCGGG-3' Reverse: 5'-CCAAAGCCTGGACCATTTGC-3'	142	XM_003127618.4
CYP11A1	Forward: 5'-CTGGAAGGAGAAGGGCACACAGAA-3' Reverse: 5'-GGCTTCTGGTAATGCTGGTGATAGG-3'	213	NM_214427.1
CYP19A1	Forward: 5'-CCAGGTTGAAGAGGAACTAATGAAGGA-3' Reverse: 5'-CATTGGGCTTGGGGAAAAACTCGAGT-3'	257	NM_214429.1
FSHR	Forward: 5'-ACGCGGTTGAACTGAGGTTTG-3' Reverse: 5'-GTAAAGCAGGTTGTTGGCCTTT-3'	191	NM_214386.3
LHCGR	Forward: 5'-GGCTGGAGTCCATTCAGACG-3' Reverse: 5'-GAAAAATTCTGCTCTTTTGTTGGCA-3'	157	NM_214449.1
VEGF	Forward: 5'-CTGCTCTACCTCCACCATGCCAAGT-3' Reverse: 5'-CAGGGGCACACAGGACGGCTT-3'	195	NM_214084.1
18s rRNA	Forward: 5'-GACACGGACAGGATTGACAGATT-3' Reverse: 5'-GAGCCAGTCAGTGTAGCGCG-3'	270	NR_046261

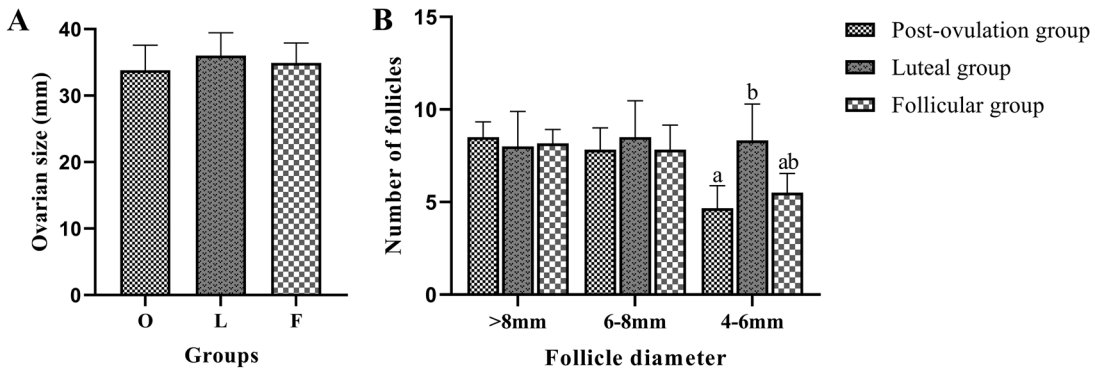

Fig. 2. Ovarian size (A) and the number of follicles (B) in gilts subjected to protocols of ovulation synchronization using altrenogest on different days of the estrous cycle. The ovaries were collected from 6 gilts in each group before administration of GnRH, A) the effect of altrenogest synchronization on ovarian size. B) the effect of altrenogest synchronization on the number of follicles. Different letters above the error bar represent significant differences ($P < 0.05$) and the same letters represent no significant differences ($P > 0.05$).

Table 2. Effect of FTAI on follicular ovulation

Time point	Groups	Number of corpora hemorrhagic	Number of recovered embryos	Number of gilts
5 h after the 2nd AI	Post-ovulation	33.00 ± 8.79 ^{ab}	20.25 ± 2.06 ^{a}	4
	Luteal	**43.75 ± 4.64** ^{b}	**37.50 ± 4.20** ^{b}	4
	Follicular	26.00 ± 11.34 ^{a}	17.25 ± 10.40 ^{a}	4

Different letters in the same column represent significant differences ($P < 0.05$) and the same letters in the same column indicate no significant differences ($P > 0.05$).

cm, n = 6) groups ($P > 0.05$; Fig. 2).

The number of follicles with sizes of > 8 mm, 6–8 mm, and < 4 mm were also not significantly different ($P < 0.05$) among the groups. However, the number of follicles with a size of 4–6 mm was significantly higher in the L group (8.33 ± 1.97, n = 6) when compared to that of the O group (4.67 ± 1.21, n = 6), but not significantly different from that of the F group (5.5 ± 1.05, n = 6) (Fig. 2).

To examine the corpora hemorrhagica and recover the early embryos and unfertilized oocytes, gilts from each group were slaughtered at 5 h after the second AI. Table 2 lists the number of ovarian corpora hemorrhagica and recovered embryos, mainly from the fallopian tubes. We found that the number of corpora hemorrhagica in the L group (43.75 ± 4.64, n = 4) was significantly higher than that in the F group (26.00 ± 11.34, n = 4; $P < 0.05$). Furthermore, the number of recovered embryos and unfertilized oocytes in the L group (37.50 ± 4.20, n = 4) was also significantly higher than that in the F

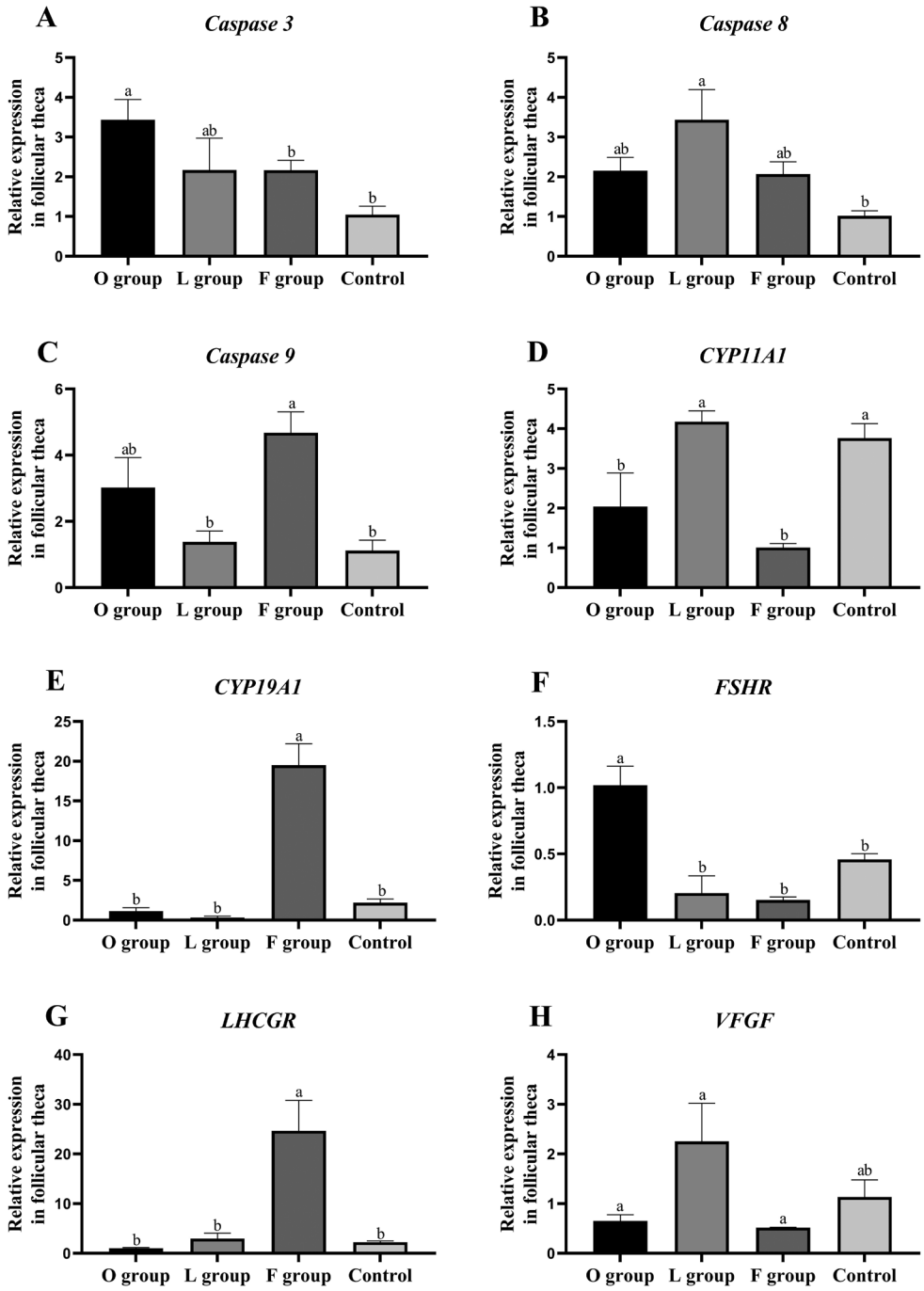

Fig. 3. The relative expression level of genes in follicular theca with a diameter of 6–8 mm. RT-qPCR was used to analyze the relative expression levels of genes. Panels (A), (B), and (C) show the relative expression levels of apoptosis associated genes *Caspase 3*, *Caspase 8* and *Caspase 9*. Panels (D) and (E) show the relative expression levels of members of the cytochrome P450 superfamily, namely *CYP11A1* and *CYP19A1*. Panels (F) and (G) show the relative expression levels of two hormone receptors, *FSHR* and *LHCGR*. Panel (H) shows the relative expression levels of *VEGF*. Different letters above the error bar represent significant differences ($P < 0.05$), the same letters represent no significant difference ($P > 0.05$) ($n = 6$ for each group).

(17.25 ± 10.40, n = 4) and O (20.25 ± 2.06, n = 4; $P < 0.05$) groups. Although there was no significant difference when compared to the O group (33.00 ± 8.79, n = 4; $P > 0.05$; Table 2), an average of 10 more corpora hemorrhagica were observed in the L group in this experiment. Otherwise, two of the gilts in the F group and one of the gilts in the O and L groups did not ovulate but exhibited abnormally enlarged follicles. Moreover, one other gilt from the O and L groups did not ovulate and had normal follicle size.

The effect of altrenogest synchronization on gene expression levels in the ovarian follicles

The theca from follicles 6–8 mm in size was collected and gene expression levels were analyzed by quantitative PCR. As shown in Fig. 3, the transcription level of follicle-stimulating hormone receptor (*FSHR*) in the O group was significantly higher than that in the L, F, and control groups ($P < 0.05$). There were no significant differences in *FSHR* transcription levels among the L, F, and control groups (P

> 0.05). Cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*) and vascular endothelial growth factor (*VEGF*) transcriptional levels were also significantly higher in the L group when compared to the O and F groups ($P < 0.05$), but not significantly different when compared to the control group ($P > 0.05$). However, the O and F groups exhibited no significant difference in *CYP11A1* and *VEGF* transcription levels ($P > 0.05$). The transcription levels of *caspase-3*, *caspase-8*, and *caspase-9* were also upregulated to a certain extent when compared to the control group and caspase-3 transcription levels in the O group were found to be significantly higher than those in the control group ($P < 0.05$) but not significantly different from those in the L or F groups ($P > 0.05$). *Caspase-8* transcription levels in the L group were also found to be significantly higher than those in the control group ($P < 0.05$) but not significantly different from those in the O or F groups ($P > 0.05$). Moreover, caspase-9 transcription levels were significantly higher in the F group than in the L and control groups ($P < 0.05$) but not the O group ($P > 0.05$). Finally, luteinizing hormone/chorionic gonadotropin receptor (*LHCGR*) and cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*) transcription levels were significantly higher in the F group when compared to those in the other groups ($P < 0.05$), while there were no significant differences among the L, O, and control groups ($P > 0.05$).

Discussion

In a normal estrous cycle, small- (1–2 mm) and medium-sized (2–9 mm) follicles are predominant in the ovaries during the luteal phase but their numbers decrease dramatically in the follicular phase, with only approximately 20 follicles maturing to ovulation follicles [24]. Notably, a greater proportion of follicles with diameters > 6 mm are ovulated compared to follicles smaller in size [25]. Thus, the number of large-sized follicles can be used to estimate litter size. In this study, we found no significant difference among the tested groups in the number of large-sized (>6 mm) follicles in gilts at 80 h after PMSG administration. However, the number of medium-sized follicles with a diameter of 4–6 mm in the L group was significantly higher than that in the O group and was slightly higher than that in the F group. Although there was no significant difference in the number of large follicles among groups, when we slaughtered the animals after the second AI, we found that the number of corpora hemorrhagica in the ovaries and the number of recovered early embryos and unfertilized oocytes in the fallopian tubes in the L group were significantly higher than those in the O group. Previous studies have clarified that the development of a follicle up to a diameter of 4–5 mm depends on gonadotrophins [26, 27]. Then, follicles reaching approximately 5 mm in diameter begin to express LH receptors in the granulosa layer, finally inducing ovulation [28, 29]. In this experiment, the number of 4–6 mm follicles in the L group significantly increased after PMSG treatment, resulting in a significant advantage in terms of the number of developing follicles larger than 4 mm among the three treatment groups. Therefore, the difference in the number of 4–6 mm follicles observed here may be one of the reasons for the increased ovulation in the L group. Otherwise, our results showed that initiation of the FTAI protocol at different estrous phases had no effect on ovarian size.

In mammalian ovaries, over 99% of follicles undergo a degenerative process known as “atresia” and only a few follicles ovulate during ovarian follicular development. Follicle atresia is mainly caused by granulosa cell apoptosis, a process that involves caspases-3, -8, and -9, as well as other cysteine proteases [30, 31]. Apoptosis is the cellular mechanism underlying ovarian follicular atresia, and the major downstream effector of this process in many tissues is caspase-3, which is expressed in the granulosa cells of the atretic follicles and is occasionally detected in healthy antral follicles [32]. Moreover, studies have shown that granulosa cells of early atretic and progressed atretic follicles exhibit significantly increased caspase-9 mRNA expression levels [33]. In this study, the expression of caspase-3 and caspase-8 in the theca of gilts in each group appeared to be upregulated to different degrees, but there were no significant differences among the groups. This suggests that the activation time and transcription levels were mostly uniform among all groups despite the initiation of the FTAI protocol during different estrous cycle phases. Thus, the higher caspase-3 and caspase-9 expression levels observed in the follicles of the F and O groups were more likely to induce cell apoptosis. In contrast, the low caspase-9 expression levels observed in the L group may be an indicator of the increase in ovulatory follicles after AI in the gilts in this group.

VEGF is essential for angiogenesis and the generation of healthy ovulatory follicles and corpora lutea. VEGF-inhibited ovaries have a restricted vasculature, increased incidence of activated caspase-3 staining, and morphological features resulting in the formation of degenerative non-functional cysts [34]. Moreover, VEGF promotes the development of follicles and reduces the formation of atretic follicles [35]. FSHR is required for VEGFA function at all follicular developmental stages [36]. In this study, although the expression of *FSHR* in the L and F groups was lower than that in the O group, it was not significantly different from that in the control group. Moreover, the observed higher *VEGF* expression levels in the L group may have provided a better environment for the development of ovulatory follicles than in the O and F groups. This may also be one of the reasons for the significant increase in the number of ovulations in the later stages of follicular development in the L group.

Owens *et al*. reported that 20% of follicles from women with polycystic ovary syndrome (PCOS), which is the most common cause of anovulation, exhibit higher LHCGR and CYP19A1 expression levels and lower FSHR and CYP11A1 expression levels [37]. In this study, at 80 h after PMSG treatment at different estrus phases, *LHCG* and *CYP19A1* transcription levels in the F group were found to be significantly higher than those in the other groups. *CYP11A1* transcription levels were also found to be significantly lower in the F and O groups when compared to the L and control groups. This suggests that when FTAI was performed at the O and F phases, the abnormal expression of *LHCGR*, *CYP19A1*, and *CYP11A1* may have affected ovary follicle development and ovulation. In contrast, the relative downregulation of *LHCGR* and *CYP19A1* and upregulation of *CYP11A1* in the L group may be helpful for ovary follicle development and ovulation.

In conclusion, initiation of the FTAI protocol during the luteal phase was shown to lead to better follicular development and ovulation. Moreover, we identified several differences in the relative expression of genes associated with follicular growth, ovulation, and apoptosis, which are as follows: compared to the O and F groups, 1) the L group had relatively lower expression levels of caspase-9, which is an apoptosis initiator and may induce the follicles to become atretic; 2) the L group has a relatively higher *VEGF* expression levels, which is essential for the generation of ovulatory follicles; 3) the L group exhibited relative *LHCGR* and *CYP19A1* downregulation and relative *CYP11A1* upregulation, which can influence follicle ovulation. However, future studies should further investigate the fertility performance of gilts, including the total litter size, live litter size, and birth weight of offspring, when using an FTAI protocol

initiated at different stages of follicular development.

Conflict of interests: The authors declare no conflict of interest.

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