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Original Article

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Abstract

The aim of this study was to optimise the ovarian responses using different FSH (follicle stimulating hormone) treatments in goats. Group 1 (control), the ovaries were stimulated with a total dose of 200 mg FSH per doe. The FSH injections were performed at 12-h intervals starting on day 9 after progesterone implantation. The FSH doses were decreased to 160 mg/doe and 130 mg/doe for Groups 2 and 3, respectively. The numbers of small, medium and large follicles were determined by real-time B mode ultrasonography. Ovum pick-up (OPU) was performed by laparoscopy under general anesthesia at 96, 42 and 36 h after the initial dose of FSH for Group 1, 2 and 3, respectively. The numbers of recovered cumulus-oocyte-complexes (COCs) and their quality were examined. The COCs were further matured and fertilised *in vitro*. It was found that the numbers of small- and medium-sizes follicles in Group 2 and 3 were significantly higher than those in Group 1, whereas the numbers of large follicles in Group 1 were significantly higher than those in the other groups ($p < 0.05$). There was no significant difference in oocyte recovery rates and the quality of COCs among groups ($p > 0.05$). However, the low-dose FSH (Group 3) tended to enhance oocyte recovery rates and oocyte quality compared to Group 1 ($p = 0.07$ and 0.06, respectively). Although the meiotic competence of recovered oocytes from Group 3 was similar to that of Group 1, cleavage rates were significantly greater than those in Group 1 (52.30 vs. 30.15%, respectively). In conclusion, ovarian stimulation with a low dose of FSH, related to the interval time between the first FSH injection and the OPU, improved oocyte recovery rates and the developmental competence of goat oocytes.

Keywords: FSH, goat, oocyte maturation, ovarian stimulation, ovum pick-up

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Introduction

Goats are one of the popular livestock animals in Asia, making them highly valuable for small-holder farms (Wodajo *et al*., 2020) and an alternative income source (Panin, 2000). The number of goats in Thailand has substantially increased by 62.5% (from 0.8 to 1.3 million) during 2019–2021 (DLD, 2021) and mostly they are mixed local breeds. In order to improve breeding efficiency and disease prevention, reproductive technologies are the potential tools to enhance the genetical values for local genetics (Baldassarre and Karatzas, 2004). Artificial insemination (AI) is the mostly used method for improving the genetic potential of desired males (Ciptadi *et al*., 2014). However, the genetic distributions of superior females to the offspring are limited. Therefore, other reproductive technologies, such as oestrous synchronization, ovarian control, *in vitro* embryo production (IVP) and embryo transfer are also integrated into the breeding programme. The regulation of ovarian follicular development is a complex process that is controlled by the reproductive hormones. Knowledge about follicular development and their interaction with the hormones is essential for a practical use of ARTs protocols. The development of the ovarian antral follicles is regulated by an increasing of follicle-stimulating hormone (FSH). Within the goat follicular cycle, only small numbers of follicles develop to the antral follicles and undergo ovulation (Gosden *et al*., 1993; Mao *et al*., 2002). The remaining subordinate follicles in each follicular wave become atretic follicles, resulting in the loss of reproductive potential. As the ovarian follicle is a functional unit of the ovary, ovarian stimulation requires appropriate endocrine signals, especially pituitary gonadotropins (FSH/Luteinizing Hormone: LH), which act on receptors located on the granulosa and theca cells (Knight and Glister, 2006). This stimulation facilitates the recruitment of the subordinate follicles and increases the numbers of oocytes for further use in the IVP program. Ovarian stimulation is a cooperative control of the oestrous and follicle stimulation, and FSH has been used as the main exogeneous gonadotropin hormone for increasing the number of follicles in goats (Hendriksen *et al*., 2000; Yu *et al*., 2003). In most cases, FSH is generally administered at a decreasing dose between 1 and 3 days before the end of progesterone impregnation (Lehloenya and Greyling, 2010). However, the administration of FSH is usually performed twice a day due to the short half-life of FSH (Armstrong *et al*., 1983). It was found that the different doses of FSH significantly affected oocyte recovery rates in goats (Kątska-Książkiewicz *et al*., 2004; Rahman *et al*., 2009; Lehloenya and Greyling, 2010). To ameliorate these shortcomings, the protocol for FSH treatment should be modified, especially when ovum pick-up is performed prior to ovulation to obtain a maximal recovered oocyte. Therefore, the objective of this study is to study the protocol for ovarian stimulation by reducing the dose of FSH and to optimise the ovarian responses to improve recovery and the developmental competence of goat oocytes.

Materials and Methods

Animals and treatments: This study was conducted under the permission of the Institute for Animal Care and Use Committee (IACUC) of Chulalongkorn University (approval number: 2031067). The animal facility is located at the Faculty of Veterinary Science, Chulalongkorn University, Nakhon Pathom Province, Thailand (latitude 13 °N and longitude 100 °E). Animals were fed concentrates, *ad libitum* grass and mineral blocks, with free access to water. A total of 12 mixed local breed animals were used, randomly allocated to three groups. Follicle development was controlled by impregnation with a controlled internal drug release device (CIDR-G, 0.3 progesterone, Zoetis, Berlin, Germany) on day 0, which was removed on day 11. Animals were administered a total dose of 200 mg of porcine purified FSH (Folltropin-V®, Bioniche Animal Health Canada, Belleville, Ontario, Canada) per doe (group 1). Intramuscular injection of FSH (50/ 50, 30/ 30, 20/ 20 mg) was performed at 12-h intervals starting on day 9. The low-dose FSH regimen was performed similar group 1, with the exception that the FSH dose was decreased to 160 mg/doe (50/ 50, 30/ 30 mg) and 130 mg/doe (50/ 50, 30 mg) for Groups 2 and 3, respectively.

Follicle observation: The follicles in both ovaries were evaluated via 7.5-MHz linear array transrectal B-mode ultrasonography (Honda HS 2100, Honda electronics, Aichi, Japan). Follicle diameter was measured and the follicles were counted. Follicles were classified as small (3–4 mm), medium (4–5 mm) and large (> 5 mm) (Menchaca *et al*., 2002). This process was performed within 2 h before OPU.

Oocyte recovery and quality assessment: The time the of OPU related to the dose of FSH administration (a decreasing dose regimen; 12 h intervals). The OPU was performed at 96, 42 and 36 h after the initiation of FSH treatment in Groups 1 (50/ 50, 30/ 30, 20/ 20 mg), 2 (50/ 50, 30/ 30 mg) and 3 (50/ 50, 30 mg), respectively. The animals were subjected to fasting for 24 h and denied access to water for 12 h before laparotomy OPU. Intravenous injections of xylazine hydrochloride (HCl) (0.1 mg/kg) and ketamine HCl (4 mg/kg) were used for general anaesthesia. Analgesic (4 mg/kg of phenylbutazone) and antibacterial drugs (20,000 IU/kg of penicillin–streptomycin) were given intramuscularly and laparotomy was performed as previously described (Anakkul *et al*., 2014). In brief, the does were moved to the laparoscopic table in a dorsal recumbent position with their heads tilted down. The incision was made caudally to the umbilicus. The structures of the ovaries were examined, including the corpus hemorrhagicum (CH), corpus luteum (CL) and follicles. Ovarian follicles larger than 2 mm in diameter were aspirated by a 21-gauge needle connected 10 ml syringe which filled with 3 ml of holding media (HM; HEPES buffer M199 supplemented with 1mM sodium pyruvate, 2 mM L-glutamine, 100 IU penicillin and 4 mg/ml bovine serum albumin (BSA), pH 7.6, 270-300mOsm/kg). The oocytes were then subjected to IVM. After the OPU procedure, the ovaries were washed with 0.9% normal saline solution with sodium

heparin (5 IU/ml) to remove any blood clots and to minimise ovarian adhesion. The cumulus oocyte complexes (COCs) were maintained at 38.5°C in HM for 3 h for transportation and grading before IVM. Subsequently, they were examined and divided into four categories according to the surrounding cumulus cells and the quality of the oocytes: 1) grade A: four or more layers of cumulus cells, 2) grade B: 1–3 layers of cumulus cells, 3) grade C: completely or partially denude of oocytes and 4) grade D: expanded and degenerated oocytes (Hashimoto *et al*., 1999).

In vitro maturation: As the results of oocyte recovery and oocyte grading, the best group was applied to compare with Group 1 (control group). Only grade A and grade B oocytes from Groups 1 and the better group (Group 3) were selected for IVM. The COCs were cultured in IVM medium which consisted of $NaHCO_3$-buffered M199, 10% (v/v) foetal bovine serum (FBS), 100 µM cysteamine, 10 ng/ml epididymal growth factor (EGF), 1 µg/ml 17β-oestradiol, 2 mM L-glutamine, 0.1 µg/ml recombinant human follicle-stimulating hormone (rhFSH; Organon, Bangkok, Thailand), 0.2 mM sodium pyruvate, 100 IU/ml penicillin and 50 µg/ml gentamicin, pH 7.6, 270-300mOsm/kg. Culturing was performed at 38.5 °C for 24 h in a humidified atmosphere of 5% CO_2 in air.

In vitro fertilization and embryo culture: After maturation, IVF was performed by co-incubation of oocytes and frozen-thawed sperm in $NaHCO_3$-buffered Fert-TALP medium (Parrish, 2014) containing 6 mg/ml BSA, 0.2 mM sodium pyruvate, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine, 10 µl/ml heparin, 100 IU/ml penicillin and 50 µg/ml gentamicin, pH 7.6, 270-300mOsm/kg. The frozen sperm was thawed at 37°C for 30 secs and subsequently diluted with a thawing medium (250 mM Tris, 90 mM citric acid, 70 mM fructose, 100 IU/ml penicillin and 100 mg/ml streptomycin) at a ratio of 1:1. The sperm was then incubated at 37°C for 3 mins prior to assessing progressive motility and only samples that had more than 50% progressive motility, were used for this study. The diluted frozen-thawed sperm was washed twice in Fert-TALP medium by centrifugation at 700 × g for 3 mins; the supernatant was removed and the sperm pellet was resuspended and co-incubated at 38.5°C for 24 h with the mature oocytes at a final concentration of 1×10^6 sperm/ml in a humidified atmosphere of 5% CO_2 in air. The presumed zygotes were further cultured at 38.5°C in a synthetic oviductal fluid (SOF) containing 10% (v/v) FBS, 1% (v/v) essential amino acids and 1% (v/v) nonessential amino acids (NEAA), 100 IU/ml penicillin and 50 µg/ml gentamicin in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. *In-vitro* development was evaluated on day 2 post IVF.

Oocyte staining and identification: The presumed zygotes were fixed and examined at 48 h post-insemination. Prior to fixation, the samples were incubated for 30 mins at 37°C in a glycerol-based microtubule-stabilising solution containing 25% (v/v) glycerol, 50 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM 2-mercaptoethanol, 50 mM imidazole, 4% Triton X-100 and 25 µM phenylmethylsulfonyl fluoride at pH 6.7. Subsequently, they were fixed and stored in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) until analysis. The oocytes were incubated with 1: 100 monoclonal anti-α-tubulin diluted with 0.1% (v/v) Triton X-100 in PBS-BSA for 1 h, washed in PBS-BSA and incubated in goat anti-mouse second antibody conjugated with tetramethylrhodamine isothiocyanate (TRITC, 1: 100) in PBS-BSA for 1 h. The actin microfilaments were stained by incubation in a 0.165-µM Alexa Fluor 488 phalloidin (Molecular Probes, Invitrogen, OR, USA) in PBS-BSA solution. The oocytes were washed in PBS-BSA and stained with 0.1 µg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 15 mins to label DNA. The stages of oocytes were then examined using an immunofluorescent microscope (BX51, Olympus, Tokyo, Japan); oocytes were classified as 1) germinal vesicle (GV), 2) metaphase I (MI) and 3) metaphase II (MII). Fertilisation was determined when cleaved embryos (2–4 cell-stage embryos) were found. Oocytes with chromatin dispersal were defined as degenerated oocytes.

Statistical analysis: Statistical analysis was performed using the SPSS statistical program version 22. Data was tested for normality and equal variance and expressed as mean ± standard deviation (SD). Comparisons among the numbers of follicles, percentages of oocyte recovery and the numbers of graded oocytes of the different groups were performed using one-way analysis of variance (ANOVA) and Bonferroni post-hoc test. The *Chi*-square test was used to test the differences in oocyte maturation, and cleavage between group. *P* values < 0.05 were considered statistically significant.

Results

Following ovarian stimulation, the numbers of follicular sizes of different groups are shown in Table 1. The numbers of small-size follicles in Group 3 were significantly greater than those in the other groups ($p < 0.001$). Medium-size follicles did not significantly differ between Groups 2 and 3 but were significantly different to that in Group 1 ($p < 0.001$). The numbers of large-size follicles in Group 1 were significantly greater than those in the other groups ($p < 0.001$).

This study also revealed that the ovarian responses following treatments with control dose (200 mg/doe) or low doses (160 and 130 mg/doe) of FSH did not significantly differ in terms of oocyte recovery rates and in the numbers of grade A and B oocytes (Table 2). However, the oocyte recovery rates and the numbers of graded A and B of oocytes in Group 3 tended to be greater than those in Group 1 (p = 0.07 and 0.06, respectively).

To study developmental competence of the oocytes, only oocytes recovered from Group 1 (standard/control FSH dose; 200 mg/doe) and Group 3 (low-dose FSH; 130 mg/doe) were used. The percentages of oocyte maturation were not significantly different between Group 1 and Group 3. However, the development of oocytes (maturation

rate, MII rate) in Group 3 was slightly higher than that in Group 1 (80.48 vs. 78.68%; p = 0.90). The oocytes from both groups developed to the two-cell stage after *in vitro* fertilisation and the cleavage rates of fertilised oocytes in Group 3 were significantly higher than those in Group 1 (52.30 vs. 30.15%; p = 0.04, Table 3).

Table 1 Effect of FSH on the numbers of small, medium and large size of follicles

Group	Number of follicles per category		
	Small size	Medium size	Large size
1	0.50 ± 0.83[c]	1.83 ± 0.75[b]	6.16 ± 0.75[a]
2	1.83 ± 0.75[b]	6.16 ± 0.98[a]	2.33 ± 0.81[b]
3	4.00 ± 0.63[a]	4.83 ± 0.75[a]	1.16 ± 0.75[c]

[a, b, c] Values within the same column with different superscript letters are significantly different ($p < 0.05$)

Table 2 Effect of FSH on the numbers of goats having ovulation, oocyte recovery rate and number of oocytes following classification

Group	No. of goat having ovulation	Oocyte recovery (%)	Oocyte grading/ goat	
			A+B	C+D
1	4/5	63.67 ± 21.60[a]	4.2 ± 3.27[a]	4.75 ± 2.07[a]
2	0/6	70.73 ± 20.90[a]	7.67 ± 4.32[a]	1.5 ± 2.07[a]
3	0/6	88.12 ± 13.51[a]	8.75 ± 4.16[a]	3.5 ± 4.50[a]

[a, b] Values within the same column with different superscript letters are significantly different ($p < 0.05$)

Table 3 Percentages of *in vitro* maturation and cleavage following *in vitro* fertilization

Group	No. of oocytes	Oocyte maturation (%)		Cleavage (%)
		MI	MII	
1	21	18.38[a]	78.68 ± 3.67[a]	30.15 ± 5.17[b]
3	27	16.74[a]	80.48 ± 6.38[a]	52.30 ± 10.19[a]

[a, b] Values within the same column with different superscript letters are significantly different ($p < 0.05$)

Discussion

Ovarian stimulation is a useful technique for increasing the numbers of recovered oocytes in IVP. This study revealed that the total amount of FSH could be reduced without significantly affecting the oocyte recovery rates and the quality of COCs. The low concentration of FSH (100 ng/ml) was supplemented in *in vitro* maturation showing an increasing of follicle viability and antrum formation when compared to the high concentration of FSH (1000 ng/ml) (Saraiva *et al*., 2011). The role of FSH is the regulation of growth factors which involved the follicular survival (Markstrom *et al*., 2002), induction the expression of apoptosis inhibitor protein by granulosa cells (Wang *et al*., 2003; Yu *et al*., 2003) and allowing the maximisation of the recruitment of subordinate follicles and the numbers of follicles present at the ovarian surface. FSH maintained the levels of FSH receptor expression, which keep the balancing of the follicles to FSH (Saraiva *et al*., 2011). On the other hand, the higher concentration of FSH had a decreased responsiveness of follicle to FSH by reducing the expression of mRNA of FSH receptor levels in the ovarian follicles (Xu *et al*., 1995). In previous studies, the oocyte recovery rates following OPU varied from 67–69% (Graff *et al*., 2000) and 24–82% (Abdullah *et al*., 2008). In the present study, follicles in all experimental groups were visible via ultrasonography as similar as the numbers observed by direct ovarian observation (Rahman *et al*., 2009; Souza-Fabjan *et al*., 2014). Several factors can affect the oocyte recovery rates and developmental competence, such as follicle size (Marchal *et al*., 2002) and oocyte quality (Krisher, 2004). The oocyte recovery rates in this study are similar to those found in a previous report (Gibbons *et al*., 2007), which ranged between 60 and 80%. However, the size of the observed follicles is dependent upon the dose of FSH and the interval time between the first FSH injection and the OPU. In this study, the numbers of large follicles were significantly greater in Group 1 when compared to Groups 2 and 3, most likely because of the dose of FSH and the time of the OPU. In Groups 2 and 3, the CIDR was still impregnated at the time of the OPU, whereas it was already removed from goats in Group 1. Progesterone essentially acts in the control of the emergence of the follicle wave and suppresses the development of large follicles (Bo *et al*., 1995; Mendes *et al*., 2018) via the granulosa cells (Long *et al*., 2021). In bovines, the oocyte recovery rates from the small follicles are generally greater than those obtained from large follicles due to the smaller amount and less viscous character of the follicular fluid (Seneda *et al*., 2001). In addition, the large follicles increased the intracellular pressure, which can cause the leakage of the follicular fluid and oocyte at the time of follicle puncture (Baldassarre *et al*., 1994). These results are similar to our findings that the oocyte recovery rates in

Group 1 were lower than those in the other groups. In addition, the morphological quality of COCs did not improve by using the standard dose of FSH when compared with lower-dose FSH regimens. In general, the developmental competence of the oocytes increases with advanced follicle development (Arlotto *et al*., 1996). However, the oocytes retrieved from medium follicles (≤ 5 mm) had higher numbers of good morphological COCs (grade A and B) when compared with large follicles. The expansion of cumulus cells surrounding the recovered oocytes indicated that the maturation process of these oocytes had already been initiated (Humblot *et al*., 2005) prior to the OPU. On the other hand, the compacted cumulus oocytes had the contraction between cumulus cells and oocytes (D'Alessandris *et al*., 2001) and could reach the blastocyst stage (Yuan *et al*., 2005). Although the maturation rate of the oocytes from Group 3 was not significantly different when compared with oocytes from Group 1, the developmental competence in terms of cleavage rate was significantly good. In a previous study, the oocytes from goats treated with FSH reached the MII stage within 18 to 27 h of culture (Le Gal *et al*., 1992). Indeed, optimally, fertilisation of the oocytes at the MII stage in mammalian species should occur within 10 h after the completion of meiosis. If MII stage oocytes are not fertilised in a timely fashion, unfertilised oocytes undergo aging (Lord and Aitken, 2013). Therefore, it is possible that intrafollicular pre-maturation of the goat oocytes within the large follicles, combined with a 24-h *in-vitro* maturation, may induce oocyte aging. This process changes several cell functions and has been reported to reduce fertilisation rates in several species such as humans (Goud *et al*., 1999), pigs (Kikuchi *et al*., 2000) and mice (Liu *et al*., 2009). The decreased fertilisation rate involves biochemical and functional alterations of oocyte physiology, such as the dysfunction of Ca^{2+} regulation (Miao *et al*., 2009). However, the responsibility of oocyte aging for the poor cleavage rates of oocytes recovered from Group 1 goats should be further elucidated.

In conclusion, ovarian stimulation with the low dose of FSH, related to the interval time between the first FSH injection and the OPU, increased the numbers of small- to medium-sized follicles that were suitable for oocyte collection when compared with the standard dose of FSH. The recovered oocytes could efficiently complete meiosis and fertilisation *in vitro*. It is recommended to use the low FSH dose in case the oocytes will be collected for *in-vitro* embryo production programmes.

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