Ovarian Follicular Dynamics, Ovarian Follicular Growth, Oocyte Yield, *In vitro* Embryo Production and Repeated Oocyte Pick Up in Thai Native Heifers Undergoing Superstimulation

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ABSTRACT: The objective of this study was to compare the effectiveness of the protocols for superstimulation of follicular growth in Thai native heifers. Heifers (n = 20) were randomly divided into four groups of five heifers/group. Heifers were given a single dose by i.m. administration of 100 mg Follicle Stimulating Hormone dissolved in polyvinylpyrrolidone (FSHp) at 24 h. Ovum pick up (OPU) occurred at 72 h ($F_{24}O_{72}$ protocol; Group 1) or 96 h ($F_{24}O_{96}$ protocol; Group 2), and at 36 h and OPU at 72 h ($F_{36}O_{72}$ protocol; Group 3) or 96 h ($F_{36}O_{96}$ protocol; Group 4) after follicular ablation. The dynamics of ovarian follicular growth were monitored by twice-daily ultrasonographic examinations. Blood sample collections were performed every 12 h after initiation of treatment for assessment of FSH, E2 and P4 profiles. All heifers were subjected to eight repeated sequential sessions of OPU. The follicular deviation commenced 24±5.32 h after follicular ablation in all groups. The circulatory FSH surged quickly from 24 to 36 h (>0.8 ng/ml) after follicular ablation and circulatory estrogen levels steadily increased from 36 h until OPU in all groups. At the end of the OPU sessions, the mean number of aspirated follicles/heifer/session in $F_{36}O_{72}$ protocol (Group 3) and $F_{36}O_{96}$ protocol (Group 4) were higher than in the two other groups (p<0.05). The number of cumulus-oocyte complexes (COCs), cleaved and day 8 blastocysts rates in the $F_{36}O_{72}$ protocol (Group 3) were higher than in the other groups (p<0.05). It can be concluded that a single dose i.m. administration of 100 mg FSHp at 36 h and OPU at 72 h after follicular ablation ($F_{36}O_{72}$ protocol; Group 3) was the most effective protocol for superstimulation of follicular growth for repeated OPU and subsequent *in vitro* embryo production in Thai native heifers. (**Key Words:** FSH, Follicle, OPU, COCs, IVP, Cattle)

INTRODUCTION

Currently, beef cattle production worldwide is affected from global warming and climate change (Koneswaran and Nierenberg, 2008). It has had an effect on the animal production and thus food safety and food security (Kabubo-Mariara, 2009). In view of these concerns, the genetics of native cattle is becoming increasingly important to cattle breeding programs once again, because of the need for cattle to adapt to the local environment in tropical or subtropical environments (Tomley and Shirley, 2009).

Furthermore, native cattle have higher heat tolerance, higher resistance to regional ectoparasitic diseases, and the ability to utilize low quality forages (FAO, 2006). In the last few decades, however, the numbers of the indigenous tropical cattle breeds as well as Thai native cattle have declined steeply, mainly due to lack of encouragement to maintain populations in the face of the threat posed by commercial breeds and modernization of agriculture (Department of Livestock Development of Thailand, 2011). Therefore, there is an urgent need to protect and propagate the Thai native cattle in order to increase the population in a shorter period of time, preserve the genetic resources and diversity of this and other native breeds.

An increase in the population of endangered mammalian species can benefit from modern reproductive biotechnologies including artificial insemination (AI), multiple ovulation and embryo transfer (MOET) and *in vitro* embryo production (IVP) (Galli et al., 2003). To date, the OPU method is a modern technique for recovery of cumulus-oocyte complexes (COCs) from live donors for IVP (OPU-IVP system) (Galli et al., 2001). Because of the
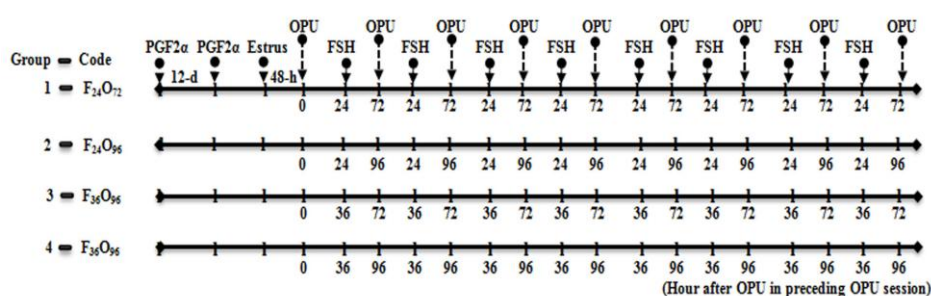
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Figure 1. Superstimulation protocols for repeated OPU in Thai native cattle.

urgent need to increase the population of native cattle in a shorter period of time, the exogenous administration of gonadotropins with OPU has become the preferred method to increase the number of follicles available for aspiration. Of the different hormones that were used, such as GnRH (Kohram et al., 1998), FSH (Reis et al., 2002), eCG (Sendag et al., 2008) and PMSG (Pieterse et al., 1992), FSH has usually given the best results in terms of number of follicles aspirated and oocytes retrieved. Furthermore, it has been used successfully in IVP from superstimulation of follicular growth with OPU techniques in Angus crossbreed cows (Chaubal et al., 2006; Chaubal et al., 2007), Holstein cows (De Roovera et al., 2005), Simmental heifers (Reis et al., 2002) and Nellore cattles (Monteiro et al., 2010).

In Thai native cattle, the number of studies on superstimulation of ovarian follicular growth and follicular aspiration is limited. Furthermore, Thai native cattle differ considerably from *B. taurus* and some breeds of *B. indicus* in ovarian follicular dynamics (Sakhong et al., 2011). Therefore, data reported for *B. taurus* or some breeds of *B. indicus* should not be extrapolated to Thai native cattle. Moreover, there is a paucity of data on the efficacy of ovarian follicular stimulation by a single dose of FSH before oocyte retrieval to avoid the stress related to repeat handling in Thai native cattle. Thus, the objective of this study was to evaluate the effectiveness of a single administration of FSH for superstimulation of ovarian follicular growth and to evaluate the best time for superstimulation and oocyte retrieval in Thai native heifers undergoing repeated superstimulation and OPU.

MATERIAL AND METHODS

Experimental animals

This study was performed at the beef cattle farm, Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand (Latitude 16°26'N, Longitude 102°50'E, altitude 163 m). During the past 30 yr, the mean annual temperature has been 27.21°C and the mean temperatures for the hottest and coldest months have been 31.53 and 21.89°C (TMD, 2005).

The animals used were nulliparous, cyclic heifers and free from reproductive abnormalities (n = 20); they were kept on pasture (*Panicum maximum*) in the winter to the rainy season from January until August, with a diet of 83% corn and 17% soybean supplementation (1 kg/head/d). The mean body weight, age and body condition score of heifers was 178.40±5.0 kg (ranging from 170 to 185 kg), 2.35± 0.15 yr (ranging from 2.2 to 2.5 yr) and 4.00±0.5 (3.5 to 4.5 on a 0 to 5 point scale) (Lowman et al., 1976).

Experimental design and superstimulation protocol

The research protocol was approved by the Animal Ethical Committee of Khon Kaen University, Thailand. In the randomized complete block design, Thai native heifers (n = 20) were divided into four groups of five heifers per group based on the average follicular numbers in the estrus cycle of each heifer from the preliminary study. Each group was allotted to a treatment protocol; all heifers were subjected to eight sequential sessions of OPU. Treatments and the experimental design are explained below and summarized in Figure 1.

Before the first aspiration, the heifers were synchronized in estrus and the dominant follicle removed according to the method previously described by Viana et al. (2010). Briefly, the heifers in all groups were synchronized in estrus by injecting 500 μg prostaglandin F2α (PGF2α) (500 μg Cloprostenol, Estrumate, Coopers, Berkhamsted, England) twice at an interval of 12 d and the estrous was observed every 12 h after last PGF2α treatment. All follicles ≥5 mm in diameter were ablated in the first OPU at a 48 h interval after estrus but before ovulation to avoid corpus luteum (CL) development and this was designated as 0 h for this experiment. The absence of a CL at the beginning of the experimental period was confirmed by ultrasonography and blood serum progesterone (P4) detection.

This first OPU was performed only to ablate the dominant follicles present at the start of the experiment and the remaining eight OPU sessions after superstimulation to detect follicular growth in each group and to evaluate the number and quality of recovered COCs. Follicular dynamics were monitored in each session of OPU. The

OPU outcomes were recorded independently, according to the development of dominant or sub-dominant follicles in the interval preceding each aspiration procedure.

Superstimulation protocol in Group 1($F_{24}O_{72}$)

Heifers were given 100 mg of FSH (Folltropin®-V) dissolved in 10 ml of 30% (wt/vol) polyvinylpyrrolidone (PVP) to form FSHp (Chasombat and Vongpralub, 2011) by a single dose administration 24 h after follicular ablation. All OPU sessions were performed at 72 h after follicular aspiration and were assigned to the $F_{24}O_{72}$ protocol code. A 24 h interval after the preceding OPU session was the time set for superstimulation of follicular growth for the subsequent OPU session over a period of eight OPU sessions.

Superstimulation protocol in Group 2 ($F_{24}O_{96}$)

In this group, the treatment regimen was the same as in Group 1, except that all OPU sessions were performed at 96 h after follicular ablation and were assigned to the $F_{24}O_{96}$ protocol code.

Superstimulation protocol in Group 3 ($F_{36}O_{72}$)

Heifers were given 100 mg of FSHp by a single dose administration at 36 h after follicular ablation; all OPU sessions were performed at 72 h after follicular ablation and were assigned to the $F_{36}O_{72}$ protocol code. A 36 h interval after the preceding OPU session was the time set for superstimulation of follicular growth for the subsequent OPU session over a period of eight OPU sessions.

Superstimulation protocol in Group 4 ($F_{36}O_{96}$)

In this group, the treatment regimen was the same as in Group 3, except that all OPU sessions were performed at 96 h after follicular ablation and were assigned to the $F_{36}O_{96}$ protocol code.

Characterization of follicular dynamics and deviation

Follicular dynamics were observed during eight consecutive aspiration intervals, beginning 12 h after each OPU session. The ovaries were examined with a portable ultrasound device equipped with a trans-rectal linear-array 7.5-MHz transducer (Scanner Honda®HS-2000, Honda Electronics Co., Ltd. Japan). The sonograms from ultrasonographic scanning of both ovaries were recorded in the ultrasound machine at examination. Evaluations were performed at the first OPU session or zero hour (h 0) of the experiment protocols and twice daily (every 12 h) after the start of the experiment over the eight OPU sessions. At each examination, the size and location of all follicles were recorded on a sketch of each ovary. The first, second, and third largest follicles were defined as F1, F2, and F3 based on individual retrospective determinations of maximum follicular diameters. Follicles with a diameter ≥7 mm were considered dominant, based on previous reports in Thai

native heifers (Chasombat and Vongpralub, 2011; Sakhong et al., 2011). The evaluations of follicular deviations were performed as previously described by Gimenes et al. (2008). Briefly, follicular deviations after follicular ablation was determined by comparing F1, F2 and F3 diameters and are designated as observed deviations. The evaluations of ovarian follicular dynamics were performed as previously described by Sartori et al. (2001). Briefly, the data of follicular dynamics after follicular aspiration were normalized to the beginning of deviation in follicular growth of F1, F2 and F3.

Follicular aspiration and oocytes recovery

The follicular aspirations were performed according to the method previously described by Bols et al. (1997). Briefly, the animals were restrained in a suitably designed stanchion, which allowed minimal movement and prepared for OPU by administering 0.02 mg/kg BW Xylazine HCL (Xylaxin, L.B.S. Laboratory Ltd., Bangkok, Thailand) i.m. followed 10 min later with an epidural anesthesia of 3 to 4 ml of 2% lignocaine HCL (Xylocaine 2%, Union drug laboratories Ltd., Bangkok, Thailand). The follicle aspiration was performed with a portable ultrasound device equipped with a sector intravaginal 7.5-MHz transducer (Honda® HS-2000, Honda Electronics Co., Ltd, Japan), and used with a 17G×490 mm sterile stainless cow ova vacuum needle (COVA Needle; Misawa Medical, Tokyo, Japan). An aspiration pump (Vakuum pumpe; Minitüb GmbH, Tiefenbach, Germany) created a vacuum of 120 mmHg and 22 ml/min aspiration rate through a disposable aspiration needle. All follicles ≥3 mm were aspirated; specific attention was given to avoid partial aspiration or completely missing a follicle. Numbers of follicles in the 2 to 3 mm, 4 to 6 mm and ≥7 mm diameter categories were recorded (Chasombat and Vongpralub, 2011; Sakhong et al., 2011). The aspirated follicular fluid was collected in 50-ml tubes containing modified Dulbecco´s phosphate buffered saline (mDPBS), supplemented with 1% fetal calf serum (Gibco) and 125 IU/ml heparin (Sigma). Recovered COCs were morphologically classified into five categories according to those previously described by Chaubal et al. (2006), as follows: Grade A, >4 layers of cumulus cells; Grade B, three or four layers of cumulus cells; Grade C, one or two layers of cumulus cells; Grade D, denuded oocytes; and Grade E, oocytes with expanded cumulus. All COCs (including Grades A-C) from each cow were used for maturation except Grades D and E which were not used for IVP.

Blood collection and hormonal assay

Blood samples were collected from the animals by coccygeal artery puncture, using 15 ml vacutainer tubes

containing heparin at zero hour of first OPU just before the start of treatment, and every 12 h after initiation of treatment over the period of eight OPU sessions in all treatment groups. Immediately after collection, the samples were centrifuged at 2,000×g for 20 min, and the plasma obtained was marked and stored at -20°C pending hormonal analysis. In order to determine possible differences between treatment group, blood samples were analyzed to determine hormone profiles of each OPU session. The analyses were performed by the double-antibody radioimmunoassay (DARIA) technique for determination of follicle stimulating hormone (FSH), progesterone (P4) and oestradiol (E2), as described previously (McNeilly and Fraser, 1987; Crowe et al., 1995; Mann et al., 1995) in the radioimmunoassay laboratory, Department of Radiology, Faculty of Medicine, Khon Kaen University, Thailand.

Plasma P4, E2 and FSH concentrations were measured without prior extraction, using an ^{125}I-label for P4, E2 and FSH DARIA (McNeilly and Fraser, 1987; Crowe et al., 1995; Mann et al., 1995). For P4, sensitivity of an assay at an 80% effective dose (ED80), inter-assay coefficients of variation (CVITE's) for low-, medium- and high-quality controls was 0.35 ng/ml, 27.3%, 19.6% and 15.7%, corresponding intra-assay coefficients of variation (CVITR's) were 17.4, 13.6 and 10.4%. For E2, ED80, CVITE's for low-, medium- and high-quality controls was 0.195 pg/ml, 20.1%, 12.4% and 14.3%, corresponding CVITR's were 12.5, 10.1 and 11.9%. For FSH, ED80, CVITE's for low-, medium- and high-quality controls was 0.05 ng/ml, 14.2%, 9.17% and 10.23%, corresponding CVITR's were 11.6, 8.3 and 9.16%.

IVM/IVF/IVC

All Grades A-C oocytes from each heifer were matured using the procedure previously described (Ratto et al., 2011). Briefly, The COCs were washed three times with mDPBS, and twice in TCM-199 (Gibco) with 10% FCS, and groups of 8-10 COCs were transferred immediately into 100 μl droplets of maturation medium of TCM-199 (Gibco), that consisted of 10% FCS, 10 μg/ml LH (Sigma), 1 μg/ml E2 (Sigma), 0.5 μg/ml FSH (Sigma) 50 IU/ml penicillin G sodium (Sigma) and 50 mg/ml streptomycin (Sigma). The droplets containing oocytes were covered with mineral oil and then the Petri dishes were placed in the incubator at 38.5°C, 5% CO_2 in air at maximum humidity for 24 h.

IVF sperm preparations were performed by the swim-up method as described previously (Parrish et al., 1995) with minor modifications. Briefly, two straws of frozen semen from a Thai native cattle bull, 0.25 ml/straw, 20 million spermatozoa/straw, were thawed in warm (37°C) water for 30 s and 200 μl of semen were placed into 2 ml of Tyrode's albumin lactate pyruvate-HEPES (TALP-HEPES)

containing 0.3% (wt/vol) bovine serum albumin (BSA; fatty acid-free) in a 15-ml tube, and incubated at 39°C, 5% CO_2 and maximum humidity. After 1 h, 1 ml of the upper fraction was collected and placed into 3 ml of TALP-HEPES, and centrifuged (2,100×g, 7 min). The pellet was then re-suspended with 3 ml TALP-HEPES and centrifugated for 7 min at 2,100×g. Afterwards the pellet was re-suspended in 150 μl fertilization medium of TALP-IVF containing 0.3% (wt/vol) fatty acid-free BSA, 3.0 μg/ml heparin (Sigma), 3.0 μg/ml penicillamine (Sigma) and the final sperm concentration was adjusted to 1×10^6/ml.

For IVF, twenty-four hours later, COCs were removed from the maturation medium and washed in TALP-HEPES medium. Excess cumulus cells were removed by gentle pipetting before groups of 10 to 15 oocytes were placed in 5 μl IVF-TALP medium and covered with mineral oil; a 95 μl suspensions of frozen-thawed spermatozoa from the IVF-TALP medium of sperm preparations was then added. Spermatozoa and oocytes were co-incubated for 18 to 20 h at 38.5°C in an atmosphere of 5% CO_2 in air at maximum humidity.

The zygotes were cultured in synthetic oviductal fluid with a minor modification (mSOF) (Holm et al., 1999). Briefly, approximately 18 to 20 h after insemination, the zygotes were washed four times in PBS and once in mSOF which consisted of 0.5 μg/ml glutamine (Sigma), 3 mg/ml BAS (G-mSOF) without glucose and citrate. Subsequently, the zygotes were transferred immediately into 100 μl droplets of G-mSOF covered with mineral oil and cultured for 28 h (d 1 to 2) at 38.5°C in an atmosphere of 5% CO_2 in air at maximum humidity. The cleavage rates were checked 48 h post insemination under stereo zoom microscopy. After that, the embryos (two cell stage or beyond) were washed twice in the mSOF which consisted of 0.5 μg/ml glucose (Sigma), 1 μg/ml citrate (Sigma), 3 mg/ml BAS (C-mSOF) without glutamine. The embryos were transferred immediately into 100 μl droplets of C-mSOF with bovine oviductal epithelial cells covered with mineral oil and cultured for another 6 d at 38.5°C in an atmosphere of 5% CO_2 in air at maximum humidity and the culture medium was replaced with fresh medium every day. Every day, embryos were examined under an inverted microscope to evaluate development. On d 8, the percentage of good quality transferable blastocysts (BL) grade 1 and 2 (Lindner et al., 1983) were recorded. The cleavage, 8-cell, 16-cell and blastocysts rate was calculated on the basis of number of oocytes cultured.

Statistical analysis

These studies were done with completely randomized designs with repeated measures (eight OPU sessions). Data

Table 1. Ovarian follicular development (Mean±SEM) after undergoing repeated superstimulation of ovarian follicular growth and OPU in Thai native heifers

Protocol code (Group)*	$F_{24}O_{72}$ (Group 1)	$F_{24}O_{96}$ (Group 2)	$F_{36}O_{72}$ (Group 3)	$F_{36}O_{96}$ (Group 4)
Number of animals (n)	5	5	5	5
Follicular population at the end of OPU sessions	15.65±2.45[a]	15.38±2.65[a]	22.75±3.21[b]	22.58±3.17[b]
Initial diameter (mm)				
*F1	3.49±0.12	3.45±0.09	3.60±0.11	3.47±0.10
*F2	2.80±0.11	2.85±0.19	2.96±0.14	2.89±0.12
*F3	2.16±0.06	2.23±0.08	2.23±0.11	2.21±0.09
Maximum diameter (mm)				
F1	5.92±0.13[c]	7.80±0.15[ab]	6.95±0.17[bc]	8.82±0.12[a]
F2	5.12±0.13[c]	5.76±0.12[b]	5.66±0.16[bc]	6.84±0.18[a]
F3	3.45±0.11[c]	3.93±0.10[b]	4.41±0.14[a]	4.45±0.10[a]
Growth rate (mm/d)				
F1	1.97±0.09[b]	1.95±0.11[b]	2.30±0.10[a]	2.20±0.08[a]
F2	1.70±0.12[a]	1.44±0.04[b]	1.88±0.06[a]	1.46±0.10[b]
F3	1.25±0.07[b]	1.00±0.03[c]	1.47±0.04[a]	1.10±0.02[c]

[a,b,c,d] Values followed by different superscripts within rows differ ($p \leq 0.05$).
*F1: largest follicles, *F2 sub-largest follicles, *F3: largest subordinated follicle. * Protocol code (Group), a single dose i.m. administration of 100 mg FSHp at 24 h and OPU at 72 ($F_{24}O_{72}$; Group 1) or 96 h ($F_{24}O_{96}$; Group 2), and a single dose i.m. administration of 100 mg FSHp at 36 h and OPU at 72 ($F_{36}O_{72}$; Group 3) or 96 h ($F_{36}O_{96}$; Group 4) after follicular ablation.

obtained during the experiments were analyzed using the mixed model procedure of Statistical Analysis System software (SAS) (SAS, 1998). The models included treatment effect and time (OPU sessions). Protected least significance differences were used to compare least square (LS) means. Significant differences between treatment groups were declared at $p \leq 0.05$.

RESULTS

Characterization of follicular dynamics and deviation

The main characteristics of the ovarian follicular dynamics after follicular ablation in all treatment groups are shown in Table 1. The total follicular numbers at the end of OPU session in $F_{36}O_{72}$ (Group 3) and $F_{36}O_{96}$ (Group 4) protocol were higher than in the other two groups ($p<0.05$). Meanwhile, there was an effect of interval between OPU sessions on maximum diameter (mm) of F1, F2 and F3 follicles ($p<0.05$); in long intervals between OPU sessions they were bigger while in short interval between OPU sessions they were smaller. The growth rates (mm/d) of F1 in $F_{36}O_{72}$ (Group 3) and $F_{36}O_{96}$ (Group4) protocol were faster than $F_{24}O_{72}$ (Group 1) and $F_{24}O_{96}$ (Group 2) protocol ($p<0.05$). In addition, there was an effect of pre-ovulatory size (F1) on growth rate (mm/d) of F2 and F3 follicles ($p<0.05$); the larger the maximum diameter of F1 follicles, the slower the growth rate in F2 and F3 follicles and vice versa.

The evaluation of the graphic representation of follicular diameter data enabled characterization of various follicular growth patterns according to F1, F2 and F3 deviations after follicular aspiration in each OPU intervals

(Figure 2). The follicle recruitment commenced at 24 h after follicular ablation in all treatment groups. Meanwhile, F1, F2 and F3 follicles >4 mm were unobserved at 24 h after follicular ablation in all treatment groups (Figure 2). Furthermore, the dominant follicle selection occurred at 48 after follicular ablation and there was a significant difference in diameter of F1, F2 and F3 ($p<0.05$) follicles among the groups at this time. After that, the F1 follicles rapidly increased in size and showed a larger maximum diameter at the end of the OPU sessions than F2 and F3 in all group treatment protocols ($p<0.05$).

Follicular aspiration and oocytes recovery

This study consisted of a single dose i.m. administration of 100 mg FSHp at 24 h and OPU at 72 h ($F_{24}O_{72}$ protocol; Group 1) or 96 h ($F_{24}O_{96}$ protocol; Group 2) and a single dose i.m. administration of 100 mg FSHp at 36 h and OPU at 72 h ($F_{36}O_{72}$ protocol; Group 3) or 96 h ($F_{36}O_{96}$ protocol; Group 4) after follicular ablation. A total of 629, 615, 910 and 903 follicles were aspirated during 40 OPU sessions in $F_{24}O_{72}$ (Group 1), $F_{24}O_{96}$ (Group 2), $F_{36}O_{72}$ (Group 3) and $F_{36}O_{96}$ (Group 4) protocol, yielding 506, 541, 834 and 830 oocytes. The total number, mean and COC classifications are shown in Table 2. The mean number of aspirated follicles /heifer/session over a period of 8 OPU sessions in $F_{36}O_{72}$ (Groups 3) and $F_{36}O_{96}$ (Group 4) protocol were higher than $F_{24}O_{72}$ (Group 1) and $F_{24}O_{96}$ (Group 2) protocol ($p<0.05$), and no differences were shown when comparison were made between $F_{24}O_{72}$ (Group1) vs. $F_{24}O_{96}$ (Group 2) or $F_{36}O_{96}$ (Group 3) vs. $F_{36}O_{96}$ (Group 4) protocols.
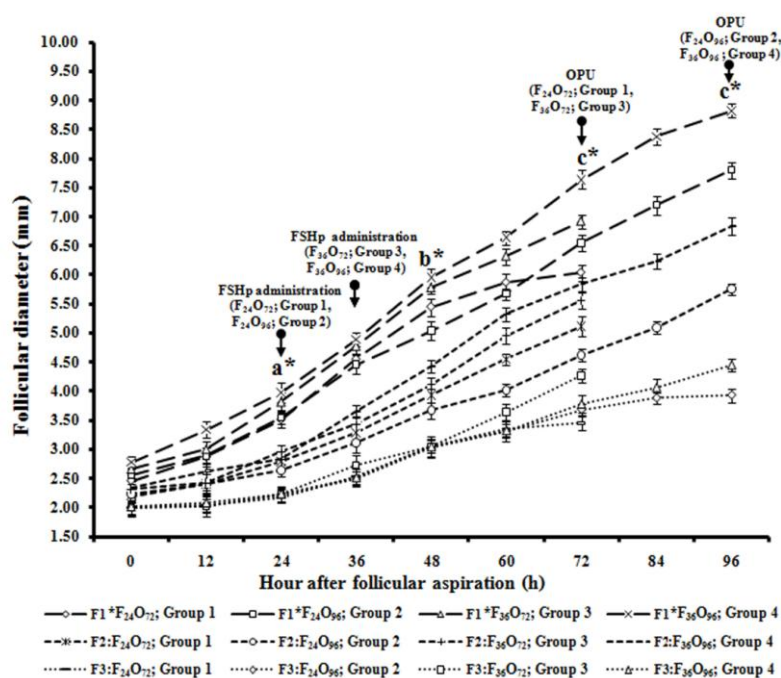
Figure 2. Ovarian follicular dynamics after follicular aspiration under repeated superstimulation of ovarian follicular growth and OPU in Thai native heifer. * Protocol code (Group), a single dose i.m. administration of 100 mg FSHp at 24 h and OPU at 72 ($F_{24}O_{72}$; Group 1) or 96 h ($F_{24}O_{96}$; Group 2), and a single dose i.m. administration of 100 mg FSHp at 36 h and OPU at 72 ($F_{36}O_{72}$; Group 3) or 96 h ($F_{36}O_{96}$; Group 4) after follicular ablation. Each dot represents mean±SEM. Follicles are identified by maximum attained diameter as F1*: largest follicles, F2* coordinate follicle and F3*: subordinated follicle. Values with asterisk (*) indicate significant differences in follicular diameter of F1, F2 or F3 at the time of beginning deviation (a), dominance follicle selection (b) and end of OPU session (c) after follicular aspiration among groups ($p<0.05$).

Table 2. Total number and mean values (Mean±SEM) of recovered COCs and classification of COCs collected undergoing repeated superstimulation of ovarian follicular growth and OPU in Thai native heifers

Protocol code (Group)*	$F_{24}O_{72}$ (Group 1)	$F_{24}O_{96}$ (Group 2)	$F_{36}O_{72}$ (Group 3)	$F_{36}O_{96}$ (Group 4)
Number of animals (n)	5	5	5	5
Characteristics				
Total number of aspiration sessions	40	40	40	40
Number of aspiration sessions (/heifer)	8	8	8	8
Total number of aspirated follicles	629	615	910	903
Mean number of aspirated follicles (/heifer/session)	15.73±2.46[b]	15.38±2.29[b]	22.75±2.56[a]	22.58±2.12[a]
Categories of follicles at aspiration (/heifer/session)				
2-3 mm	5.70±1.03[a]	0.18±0.02[c]	6.95±1.45[a]	0.68±0.06[b]
4-6 mm	9.65±1.12[b]	9.95±1.59[b]	14.20±2.17[a]	14.75±2.05[a]
>7mm	0.30±0.03[d]	5.25±0.10[b]	1.60±0.06[c]	7.15±1.04[a]
Total number of recovered oocytes	506.0	541.0	834.0	830.0
Recovery rate (%)	80.83±2.68[d]	87.97±2.89[bc]	91.65±3.03[a]	89.25±2.54[ab]
Mean number of oocytes/heifer/session	12.65±1.23[b]	13.53±1.28[b]	20.85±2.0[a]	20.75±2.04[a]
Oocytes categories				
A/Total (%)	82/506 (16.21)[b]	54/541 (9.8)[d]	222/834 (26.62)[a]	120/830 (14.46)[c]
B/Total (%)	142/506 (28.06)[a]	104/541 (19.22)[c]	221/834(26.50)[b]	145/830 (17.47)[d]
C/Total (%)	214/506 (42.29)[a]	107/541 (19.78)[c]	276/834(11.27)[d]	170/830 (20.48)[b]
D: Degenerated COCs or oocytes (%)	68/506 (13.44)[b]	112/541 (20.70)[a]	94/834(11.27)[c]	174/830 (20.96)[a]
E: Expanded COCs (%)	0/506 (0.00)[d]	164/541 (30.31)[a]	21/834(2.52)[c]	221/830 (26.63)[b]

[a,b,c,d] Values followed by different superscripts within rows differ ($p<0.05$).
*Protocol code (Group), a single dose i.m. administration of 100 mg FSHp at 24 h and OPU at 72 ($F_{24}O_{72}$; Group 1) or 96 h ($F_{24}O_{96}$; Group 2), and a single dose i.m. administration of 100 mg FSHp at 36 h and OPU at 72 ($F_{36}O_{72}$; Group 3) or 96 h ($F_{36}O_{96}$; Group 4) after follicular ablation.

The follicle size categories in all treatment groups are shown in Table 2. The number of small follicles (2 to 3mm in diameter) in $F_{24}O_{72}$ (Groups 1) and $F_{36}O_{72}$ (Group 3) protocol were higher than $F_{24}O_{96}$ (Groups 2) and $F_{36}O_{96}$ (Group 4) protocol whereas, the number of medium follicles (4 to 6 mm in diameter) in $F_{36}O_{72}$ (Groups 3) and $F_{36}O_{96}$ (Group 4) were higher than $F_{24}O_{72}$ (Groups 1) and $F_{24}O_{96}$ (Group 2) protocol (p<0.05). The largest number of larger follicles (>7 mm in diameter) was found in $F_{36}O_{96}$ (Group 4) followed by $F_{24}O_{96}$ (Group 2) protocol and both groups were higher than $F_{24}O_{72}$ (Groups 1) and $F_{36}O_{72}$ (Group 3) protocols (p<0.05).

The mean number of recovered oocytes/heifer/session in $F_{36}O_{72}$ (Groups 3) and $F_{36}O_{96}$ (Group 4) protocols were higher than $F_{24}O_{72}$ (Group 1) and $F_{24}O_{96}$ (Group 2) protocol (p<0.05), whereas, there was no difference between $F_{24}O_{72}$ (Groups 1) vs. $F_{24}O_{96}$ (Group 2) protocol and $F_{36}O_{96}$ (Group 3) vs. $F_{36}O_{96}$ (Group 4) protocol. The oocyte recovery rate, defined as the percentage of COCs recovered per total number of aspirated follicles, in $F_{36}O_{96}$ (Group 3) protocol was higher than in the other groups (p<0.05).

The distributions of oocytes according to morphological grades, as a percentage of total oocytes retrieved, are presented in Table 2. The $F_{36}O_{96}$ (Group 3) protocol generated more (p<0.05) grade A, B and C oocytes, as compared to $F_{24}O_{72}$ (Groups 1), $F_{24}O_{96}$ (Group 2) and $F_{36}O_{96}$ (Group 4) protocols. Moreover, the percentage of degenerated COCs (D) and expanded COCs (E) in $F_{36}O_{96}$ (Group 3) protocol was lower than in $F_{24}O_{96}$ (Group 2) and $F_{36}O_{96}$ (Group 4) protocol, but was higher than $F_{24}O_{72}$ (Groups 1) protocol (p<0.05), whereas, the number of COCs in Grade D and E was higher in $F_{36}O_{96}$ (Group 4) protocol than in the other groups (p<0.05).

Hormone profile

The hormone profiles of FSH, E2 and P4 after follicular aspiration following superstimulation of ovarian follicular growth and repeated OPU throughout eight OPU session periods are presented in Figures 3A, 3B. As shown in Figure 3A, the concentrations of FSH remained low (<0.6 ng/ml) until 24 h after follicular aspiration in all treatment groups. In fact, at 24 h after follicular aspiration, the circulatory FSH concentrations in the $F_{24}O_{72}$ (Groups 1) protocol were lower than in any other three Groups (p<0.05). In $F_{24}O_{72}$ (Groups 1)and in the $F_{24}O_{96}$ (Group 2) protocol after FSHp treatment the circulatory FSH concentrations sharply increased until 72 h (Group 1) or 96 h (Group 2). Nevertheless, the maintenance level was lower than in $F_{36}O_{96}$ (Group 3) or $F_{36}O_{96}$ (Group 4) protocol (p<0.05).

The circulatory FSH concentrations surged quickly from 24 to 36 h (>0.8 ng/ml) (Figure 3A) after follicular ablation in all group treatment protocols. At 36 h after follicular

ablation, the concentration of FSH in $F_{36}O_{96}$ (Group 4) protocol was higher than any other three group (p<0.05). Moreover, after FSHp administration the circulatory FSH concentrations were sharply increased and were maintained at a higher level in $F_{36}O_{96}$ (Group 3) and $F_{36}O_{96}$ (Group 4) protocols than in $F_{24}O_{72}$ (group 1) and $F_{24}O_{96}$ (Group 2) protocols (p<0.05).

The estrogen concentrations in plasma were maintained at a lower level (<0.08 pg/ml) 12 to 36 h after follicular ablation in all treatment protocol groups (Figure 3B). Thereafter, there were sharp increases from 36 h until OPU in all treatment protocol groups corresponding with the beginning of follicular deviation at 24 h after follicular ablation (Figure 2). Nevertheless, the circulatory levels of E2 from 36 to 72 h in the $F_{36}O_{72}$ protocol (Group 3) were higher than in $F_{24}O_{72}$ protocol (Group 1) but were maintained at a level lower than in $F_{36}O_{96}$ protocol (Group 4) (p<0.05). Meanwhile, the circulating levels of E2 from 36 to 96 h in $F_{36}O_{96}$ (Group 4) protocol were higher than in the other groups (p<0.05). In fact, the circulating levels of E2 from 36 to 76 h in $F_{24}O_{72}$ (Group 1) or 24 to 96 h in $F_{24}O_{96}$ (Group 2) protocol were maintained at a lower level than in the any of the other groups (p<0.05). Meanwhile, the circulating P4 concentrations were maintained at <0.5 ng/ml in all treatment protocol groups throughout the experimental period (Figure 3B).

***In vitro* embryo production**

The results of *in vitro* embryo production are presented in Table 3. The cleavage rates and day 8 blastocysts rate/oocytes or zygotes in the $F_{36}O_{72}$ (Group 3) protocol were higher than the other three groups (p<0.05), while COCs in $F_{24}O_{96}$ (Group 2) protocol showed lower efficiency for *in vitro* embryo production in terms of cleavage rates and day 8 blastocysts rates than in the other groups (p<0.05). In addition, COCs in the $F_{24}O_{72}$ (Group 1) protocol showed higher efficiency for *in vitro* embryo production in terms of cleavage rates expressed as day 8 blastocysts rates/oocytes or zygote than either $F_{24}O_{96}$ (Group 2) or $F_{36}O_{96}$ (Group 4) protocols (p<0.05)**.**

DISCUSSION

There was poor documentation of ovarian follicular dynamics, oocyte yields and *in vitro* embryo production in Thai native heifers undergoing superstimulation of ovarian follicular growth and repeated OPU. In this study, sequential monitoring of follicular dynamics after superstimulation of ovarian follicular growth and repeated OPU sessions enabled characterization of follicular wave emergence, follicular deviation and its detrimental effects on quality of recovered COCs and subsequent *in vitro* embryo production in Thai native heifers. An important
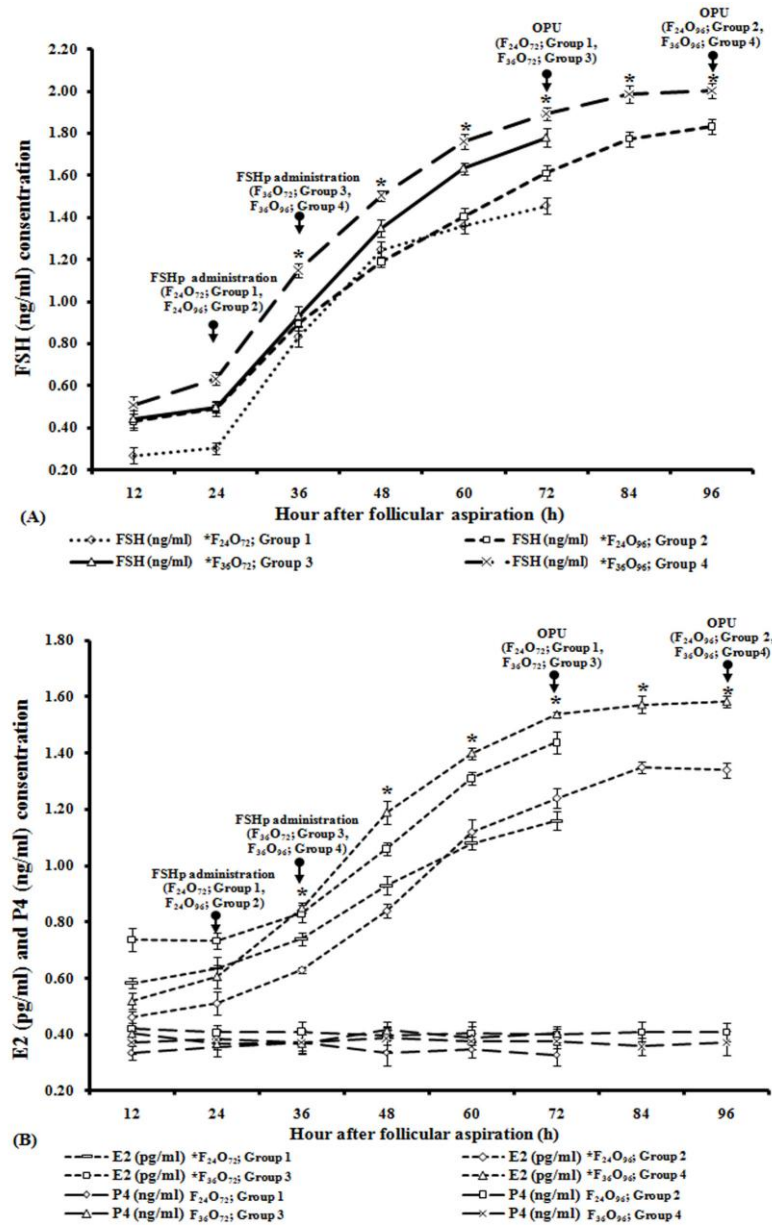
Figure 3. Plasma FSH (Figure 3A), Oestradiol-17β (E2) and Progesterone (P4) (Figure 3B) concentrations after follicular aspiration undergoing repeated superstimulation of ovarian follicular growth and OPU in Thai native heifer. * Protocol code (Group), a single dose i.m. administration of 100 mg FSHp at 24 h and OPU at 72 ($F_{24}O_{72}$; Group 1) or 96 h ($F_{24}O_{96}$; Group 2), and a single dose i.m. administration of 100 mg FSHp at 36 h and OPU at 72 ($F_{36}O_{72}$; Group 3) or 96 h ($F_{36}O_{96}$; Group 4) after follicular ablation. Each dot represents mean±SEM. Values with asterisk (*) indicate significant differences in FSH concentration (Figure 3A) or E2 (Figure 3B) at the time of observation during OPU sessions after follicular aspiration among groups (p<0.05).

Table 3. *In vitro* fertilization and embryo development (Mean±SEM) on repeated superstimulation of ovarian follicular growth and OPU in Thai native heifers

Protocol code* (Group)	No. Oocytes culture (categories A, B and C)	Cleaved zygote (n) 2-cell	Cleaved zygote (n) ≥4	Cleavage rate (%)	Cleaved /heifer/session (n)	Day 8 blastocysts (n)	Day 8 blastocysts rate /Oocytes (%)	Day 8 blastocysts rate /Zygote (%)	Blastocysts/hei fer/session (n)
$F_{24}O_{72}$ (Group 1)	438.00	237.00	141.00	86.30[b]	9.45±1.24[b]	140.0	31.96±1.03[b]	37.03±1.06[b]	3.50±0.39[b]
$F_{24}O_{96}$ (Group 2)	265.00	136.00	80.00	81.50[d]	5.40±1.32[c]	65.0	24.53±1.78[d]	30.09±2.32[d]	1.63±0.45[c]
$F_{36}O_{72}$ (Group 3)	719.00	440.00	234.00	93.74[a]	16.58±1.23[a]	272.0	37.83±2.45[a]	40.39±1.58[a]	6.80±0.79[a]
$F_{36}O_{96}$ (Group 4)	435.00	253.00	115.00	84.60[c]	9.20±1.17[b]	126.0	28.96±1.48[c]	34.24±1.02[c]	3.15±0.37[b]

[a,b,c,d] Values followed by different superscripts within rows differ (p<0.05).
* Protocol code (Group), a single dose i.m. administration of 100 mg FSHp at 24 h and OPU at 72 ($F_{24}O_{72}$; Group 1) or 96 h ($F_{24}O_{96}$; Group 2), and a single dose i.m. administration of 100 mg FSHp at 36 h and OPU at 72 ($F_{36}O_{72}$; Group 3) or 96 h ($F_{36}O_{96}$; Group 4) after follicular ablation.

finding from this study was showed the follicular development in terms of numbers and size of follicles was influenced by the different times of a single dose FSHp administration after follicular ablation. Furthermore, the exogenous FSHp treatment for inducing follicular development influenced follicular growth rate, follicular size and follicle numbers. Moreover, the different times at which oocytes were harvested between OPU intervals influenced COCs quality and embryo production. The results showed that the follicle numbers were positively correlated with collected and cultured COCs and Day 8 blastocysts. In addition, the largest follicles (>7 mm in diameter) were negatively correlated with COC quality.

In the present study, the new follicular waves emerged within 24 h after ablation of all follicles ≥3 mm in diameter. In contrast, it has been report that the emergence of new follicular waves occurred within 48 h in Gyr breed cows (Viana et al., 2012) and Holstein-Friesian heifers (Garcia and Salaheddine, 1998) after removal of all follicles ≥3 mm in diameter. This indicated that the new follicular wave emergence after follicle ablation in Thai native heifers was earlier than in those breeds. Furthermore, the investigation of FSH profiles in this study showed a steady increase of FSH and culminating in a surge at 24 to 36 h after follicular ablation. In previous studies it was reported that removal of a dominant follicle resulted in an increased FSH concentration within 5 h and an endogenous FSH surge about 1.5 d later (Ginther et al., 1999), initiating a new follicular wave within 2 d (Ginther et al., 2001). The reasons for these differences between Thai Native heifers and those other breeds is difficult to explain, but may be due to the differences in follicular development between breeds (Chaubal et al., 2006).

In the present study, deviation of follicular growth into F1, F2 and F3 occurred at 24 h after follicular ablation; at this time of follicular deviation the diameters of the dominant follicles (F1) were approximately 4.0±0.4 mm and corresponded with time of endogenous FSH surge after follicular aspiration. Moreover, the selection of the dominant follicle occurs when the largest follicle achieves 5.5±0.2 mm in diameter. This supported the idea that establishment of dominant follicles, was due to rapid follicular growth. Furthermore, the differences in size and number of large follicles (>7 mm in diameter) also corresponded to the differences in E2 production between groups. Meanwhile, a previous study has reported that the diameter of dominant follicle at selection was 6.2±0.3 mm in Gry cows and 8.5±0.3 mm in Nellore and Holstein-Friesian heifers (Garcia and Salaheddine, 1998; Castilho et al., 2007). While, the deviation of follicular growth commenced at 48 h, 36 to 48 h and 48 to 96 h in Gry cows (Viana et al., 2012), Nellore heifers (Castilho et al., 2007) and Holstein-Friesian heifers (Garcia and Salaheddine,

1998) after follicular aspiration. This finding indicated that the diameter of the dominant follicle at deviation was smaller and the time of beginning of follicular deviation and the time of FSH surges after follicular aspiration in Thai native heifers were shorter than those previously reported in other breeds.

In the present study, induced follicular growth by a single dose administration of FSHp at 36 h in $F_{36}O_{72}$ (Group 3) and $F_{36}O_{96}$ (Group 4) after follicular ablation resulted in a higher ovarian response than in the $F_{24}O_{72}$ (Group 1) and $F_{24}O_{96}$ (Group 2) protocols were FSHp treated at 24 h after follicular ablation in terms of numbers of large antral follicles at the end of a OPU session. At 36 h after follicular ablation, the small follicles ≥4 to 5 mm in diameter were recruited supported by the FSH surge. On the other hand, at 24 h after follicular aspiration only oocytes ≤3 mm in diameter were found and circulation of FSH was maintained at nadir levels. FSH is known to stimulate development of follicles starting at a diameter of 4 to 5 mm and reestablishment of a pool of large antral follicles corresponding with an FSH surge (Webb et al., 1999). Ginther et al. (2002) reported that the beginning of depression of the FSH receptors (FSHr) expressed in small follicles >4 mm in diameter when they grow into larger diameter size promotes estradiol synthesis by the follicles. In fact, in the current study, the circulation levels of FSH and E2 from 36 to 72 h after follicular aspiration in $F_{36}O_{72}$ (Groups 3) and $F_{36}O_{96}$ protocols (Group 4) were higher than $F_{24}O_{72}$ (Group 1) and $F_{24}O_{96}$ (Group 2) protocol. FSH stimulation of granulosa cell proliferation is essential for the follicular and oocytes cell growth and development during the early antral stages (Ginther et al., 2002; Adriaens et al., 2004). Meanwhile, estrogen stimulated FSH receptor expression in primary and secondly bovine oocytes in granulosa cells (Hunzicker-Dunn and Maizels, 2006). It was directly linked to stimulation of folliculogenesis by synchronization of the follicular waves to increase the number of pre-antral follicles (Nakano et al., 1982). This may be the reason for a high ovarian response and a larger population of large antral follicles in $F_{36}O_{72}$ (Group 3) and $F_{36}O_{96}$ (Group 4) protocols. The number of follicles, leaving this growing pool and becoming FSH-sensitive depends, however, on other factors, such as insulin-like-growth factor (IGF), epidermal growth factor (EGF) (Nakano et al., 1982; Gutierrez et al., 2000) and the size of the growing pool (Cushman et al., 1999).

In the present study, the smaller diameter of the dominant follicle at deviation was consistent with the smaller final diameter of the dominant follicles in Thai native heifers. Furthermore, the establishment of dominant follicles during 72 or 96 h inter-OPU intervals may be due to the apparent faster growth rate of F1 than F2 and F3 as reported for Nellore (Sartorelli et al., 2005; Gimenes et al.,

2008) and Gyr cows (Viana et al., 2004; Viana et al., 2010). However, the initial diameters of F1, F2 and F3 among intervals between OPU sessions in all groups were similar. The large follicles in $F_{24}O_{96}$ (Groups 2) and $F_{36}O_{96}$ (Group 4) of OPU at 96 h following follicular ablation were due to the longer time for growing than the other two groups. Therefore, these groups showed higher maximum diameter and a larger population of large follicles (>7 mm in diameter) at the end of OPU session than in the other groups. Furthermore, there was an effect of growth rate of F1 follicles on F2 and F3 growth; faster the growth of F1, the lower the growth rate of F2 and F3. This indicated that the F1 suppressed growth rate of F2 and F3 (Ginther et al., 2001).

In the present study, the percentage of COCs Grade A, B and C in the protocols in which OPU was performed at 72 h after follicular aspiration ($F_{24}O_{72}$; Group 1 and $F_{36}O_{72}$; Group 3) was higher than in the protocols in which OPU was performed at 96 h ($F_{24}O_{96}$; Group 2 and $F_{36}O_{96}$; Group 4). On the other hand, the percentage of COCs graded D and E was higher in the groups in which oocytes were harvested at 96 h than in the protocols in which OPU was performed at 72 h after follicular ablation. This implies that the interval between OPU sessions have an effect on COCs quality. The reason for this difference may be due to the 96 h interval between consecutive OPU sessions that allows the development of a dominant follicle which probably exerts a deleterious influence on oocytes development (Lucy, 2007). These oocytes are reported to be of lower quality and have already undergone cumulus expansion and atresia (Bergfelt et al., 1994; Bols et al., 1998). This may be the reason for the lower embryo development rate from the oocytes retrieved at 96 h after follicular aspiration. Therefore, this can be attributed to the aspiration of a newly recruited pool of follicles 3 d following the previous aspiration, that prevented establishment of a dominant follicle and regression of subordinate follicles, resulting in a more homogeneous cohort of oocytes (Bergfelt et al., 1994; Bols et al., 1998).

In the present study, there was a slower embryo development rate from the oocytes retrieved at 96 h after follicular aspiration than those recovered at 72 h. It is speculated that this may be due to the longer period of suppression of the growth of small and medium follicles by a predominant follicle or the largest follicle (>7 mm in diameter) than in the other group. In contrast, in the large follicles the follicular environment may permit greater storage of maternal mRNA and proteins within the oocyte, enhancing its capability of developing to the blastocyst stage. Furthermore, the higher developmental potential of oocytes originating from larger follicles has been attributed to the growth factors present in larger follicles (Lonergan et al., 1994), increased nuclear maturation (Fuhrer et al., 1989)

and better cytoplasmic maturation (Ectors et al., 1995). In fact, in the present study, the largest follicle (>7 mm in diameter) in the protocol of oocytes harvested at 96 h ($F_{24}O_{96}$; Group 2 and $F_{36}O_{96}$; Group 4) was larger than the protocols of oocytes harvested at 72 h ($F_{24}O_{72}$; Group 1 and $F_{36}O_{72}$; Group 3) after follicular aspiration. An embryo development rate in these protocols was slower than the protocols in which OPU was performed at 96 h after follicular ablation. This study does not separate follicle size into categories (large, medium, and small) for the investigation of embryo development from different follicle size in *in vitro*. Therefore, the influence of follicle size on embryo development in this case is difficult to explain. This may be because follicle size alone cannot be the only major influence on developmental competence (Sirard and Blondin, 1996).

In the present study, heifers in the protocols in which OPU was performed at 96 h after follicular aspiration (Group 2 and 4) showed higher circulatory levels of FSH and E2 concentrations at the end of OPU sessions in comparison with the groups in which OPU is carried out at 72 h post-follicular ablation irrespective of the mode of FSH treatment ($F_{24}O_{72}$; Group 1 vs $F_{24}O_{96}$; Group 2, $F_{36}O_{72}$; Group 3 vs $F_{36}O_{96}$; Group 4). Furthermore, it was correlated with higher number of large follicle (>7 mm in diameter) in these groups. The quality of oocytes retrieved from $F_{24}O_{96}$ (Group 2) and $F_{36}O_{96}$ (Group 4) protocols, however, was lower than in $F_{24}O_{72}$ (Group 1) and $F_{36}O_{72}$ (Group 3) protocols in term of embryo development. The reason for this difference may be attributed to the higher number of pre-ovulatory follicles at the end of OPU sessions in these groups than the other two groups. In cattle the pre-ovulatory follicle contain high amounts of FSH, estrogen and IGF resulting in an increased concentration in follicular fluid for maintenance of healthy oocytes and their survival (Kastelic et al., 1990; Turzillo et al., 1993). Conversely, the concentrations of these hormones were decreased and lower concentrations were found in the subordinate follicles than in pre-ovulatory or the largest follicle at the time of deviation and selection (Kastelic et al., 1990; Turzillo et al., 1993; Mihm et al., 2000). In fact, FSH, estradiol and IGF are known as survival factors of ovarian follicles (Monniaux and Pisselet, 1992; Turzillo et al., 1993; Mihm et al., 2000), and therefore the decrease in these hormones in subdominant follicles (small and medium follicle) leads to disruption of receptor expression of these hormones in granulosa cells. This event signals induction of the Apoptosis-Inducing Factor (AIF) such as Bcl-2 family protein expressions (Tilly et al., 1995; Dhanabal et al., 1999). The Bcl-2 family includes Bax, Bab that are pro-apoptotic proteins and Bcl-2; Bcl-x-long is an anti-apoptotic protein (Tilly et al., 1995; Dhanabal et al., 1999). It have been reported that the Bax was very high and Bcl-2 was at

very low levels in small and medium follicles but it was just the opposite in largest or pre-ovulatory follicle at the time of selection and deviation (Chao and Korsmeyer, 1998). Therefore, the small and medium follicles were may be undergoing degeneration or atresia. This is consistent with the results of the present study because the groups of heifers OPU at 96 h after follicular aspiration showed a larger number of degenerate oocytes than other Groups at the end of OPU sessions.

In conclusion, a single dose administration of FSHp at 36 h and oocytes harvested at 72 h after follicular aspiration ($F_{36}O_{72}$ protocol; Group 3) was the best protocol for superstimulation of ovarian follicular growth and repeated OPU and subsequent *in vitro* embryo production in Thai native heifers. Heifers were successfully stimulated repeatedly and oocytes harvested in eight OPU sessions, using a single dose i.m. administration of 100 mg FSHp. This protocol gave a higher ovarian response, produced good quality COCs and higher embryo production *in vitro*. Future studies could explore the integration of OPU, *in vitro* embryo production and embryo transfer on a large-scale for increasing population numbers and integration of these technologies into animal breeding programs using Thai native cattle (*B. indicus*) donors.

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