

—Technology Report—

Superstimulation of Follicular Growth in Thai Native Heifers by a Single Administration of Follicle Stimulating Hormone Dissolved in Polyvinylpyrrolidone

Jakkhaphan CHASOMBAT¹⁾, Denpong SAKHONG¹⁾, Takashi NAGAI²⁾, Rangsun PARNPAI³⁾ and Thevin VONGPRALUB¹⁾

¹⁾Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand

²⁾NARO Institute of Livestock and Grassland Science, Ibaraki 305-090, Japan

³⁾Embryo Technology and Stem Cell Research Center and School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

Abstract. This study was undertaken to determine whether a single i.m. injection of FSH dissolved in 10 ml of 30% (wt/vol) polyvinylpyrrolidone (PVP; MW=40,000) to form FSHp would induce follicular growth in Thai native heifers and to determine its optimal dose. In Group 1, heifers (n=4) were given multiple i.m. injections of FSHp every 12 h for 3 days at decreasing doses, for a total of 100 mg (control). In Groups 2, 3 and 4, heifers (n=4 in each group) were given single i.m. injections of FSHp at 50, 100 and 150 mg. All heifers received a single injection of 750 µg PGF2α 48 h after the initiation of exogenous FSH treatment. Ovaries of treated heifers were examined by transrectal ultrasonography every day until they showed estrus. Group 3 showed significantly higher numbers of ovulation follicles, significantly higher growth rates of follicles per day and significantly larger diameters of follicles and corpora lutea than groups 1 and 2 but not Group 4 (P<0.05). Group 4 showed significantly higher numbers of large follicles (≥5 mm in diameter), unovulated follicles and ovulations, a significantly higher growth rate of follicles per day, and significantly larger diameters of follicles and corpora lutea (P<0.05) than those of the other groups. This indicates a state of overstimulation of ovaries in this group. Besides, the plasma levels of FSH in Group 4 were significantly higher (P<0.05) than in the other group and were maintained in the range of 2.2–0.7 ng/ml over a period of 6 to 66 h after the FSHp injection. Meanwhile, the plasma levels of P4 and E2 did not differ in any of the groups in the period of 0 to 96 h during the superstimulation program. In conclusion, it was demonstrated that a single i.m. injection of 100 mg FSHp was the most effective dose for superstimulation of follicular growth in Thai native heifers under the experimental conditions in this study.

Key words: Cattle, Follicle stimulating hormone (FSH), Polyvinylpyrrolidone (PVP)

(J. Reprod. Dev. 59: 214–218, 2013)

When superovulation (SOV) and ultrasound-guided transvaginal follicular aspiration for ovum pickup (OPU) treatment are commonly performed, follicle stimulating hormone (FSH) is usually given for 4–5 days in decreasing doses to induce multiple follicular growth in cattle. To reduce labor and prevent stress due to repeated animal handling twice daily with FSH injections for superstimulation of follicular growth, alternative methods of administering FSH to cattle have also been examined. For example, a single intermuscular (i.m.) treatment of 400 mg FSH diluted in a hyaluronan-based slow-release formulation induced follicular growth [1]. Moreover, continuous FSH release by an osmotic pump implant or a single dose of FSH dissolved in aluminium gel [2] also stimulated ovarian follicular development in cattle.

Recently, various types of adsorbents for macromolecules that can achieve sustained release of compounds *in vivo* have been

developed. Polyvinylpyrrolidone (PVP) can absorb macromolecules, such as proteins or toxoids, and release them gradually [3]. A few studies have tested the efficacy of single-dose administration of FSH dissolved in PVP (pFSH) in SOV and OPU programs in cattle [4]. There have been no studies, however, that tested the efficacy of a single-dose administration of pFSH in Thai native cattle. There is also a paucity of data on the efficacy of ovarian stimulation by a single dose of pFSH before induced ovulation and oocyte retrieval in Thai native cattle. Furthermore, the previous studies on SOV in Thai native cattle using a single-dose injection of FSH not dissolved in PVP showed poor superovulatory response and embryo recovery [5, 6]. Thus, the present study investigated whether a single administration of FSH dissolved in PVP could successfully induce follicular growth in Thai native heifers and enable harvest of an acceptable number of additional follicles to optimize the number of oocytes collected by OPU.

Table 1 shows that the number of large follicles (≥5 mm in diameter), maximum follicular diameters, rate of follicular growth per day, number of ovulation follicles, number of anovulation follicles and maximum diameter of corpora lutea (CLs) in Group 4 were higher (P<0.05) than those in Groups 1, 2 and 3 respectively.

Received: July 25, 2012

Accepted: November 22, 2012

Published online in J-STAGE: December 20, 2012

©2013 by the Society for Reproduction and Development

Correspondence: T Vongpralub (e-mail: vthevi@kku.ac.th)

Table 1. Efficiency of superstimulation of follicular growth with a single administration of various doses of FSHp in Thai native heifers

Treatment group*	1	2	3	4
No. of heifers	4	4	4	4
No. of heifers in estrus (%)	4 (100)	4 (100)	4 (100)	4 (100)
No. of large follicles (diameter ≥ 5 mm)	16.50 \pm 0.29 ^b	10.00 \pm 0.41 ^c	17.50 \pm 0.65 ^b	25.25 \pm 1.03 ^a
Maximum follicular diameter (mm)	8.84 \pm 0.30 ^b	6.75 \pm 0.23 ^c	9.31 \pm 0.26 ^b	12.59 \pm 0.03 ^a
Growth rate (mm/d)	0.94 \pm 0.01 ^c	0.59 \pm 0.03 ^d	1.03 \pm 0.01 ^b	1.83 \pm 0.01 ^a
No. of ovulations (No. of CLs)	11.50 \pm 0.65 ^c	8.00 \pm 0.71 ^d	13.00 \pm 0.41 ^b	16.50 \pm 0.65 ^a
No. of unovulated follicles	5.00 \pm 0.58 ^b	2.00 \pm 0.41 ^c	4.50 \pm 0.29 ^b	8.75 \pm 0.48 ^a
Maximum diameter of CLs (mm)	9.13 \pm 0.08 ^c	7.70 \pm 0.33 ^d	10.41 \pm 0.39 ^b	12.00 \pm 0.43 ^a

Different superscripts (^{a,b,c,d}) in the same row indicate a significant difference ($P < 0.05$). *Group 1 heifers were given multiple i.m. injections of FSH every 12 h for 3 days at decreasing doses for a total of 100 mg (served as the control), and Groups 2, 3 and 4 were given single i.m. injections of FSHp at 50, 100 or 150 mg. Values are means \pm SEM.

Over a 96 h period, furthermore, the maximum diameter of CLs, follicular growth rate per day and number of ovulation follicles in Group 3 were significantly higher ($P < 0.05$) than those in Groups 1 and 2. However, there were no significant differences in the mean number of large follicles (≥ 5 mm in diameter), maximum follicular diameters and number of unovulated follicles between Group 1 and Group 3. Group 2 had the lowest ($P < 0.05$) superstimulation of follicular growth response among groups.

The present data demonstrated a dose response to a single i.m. injection of FSHp relative to the number of large follicles (≥ 5 mm in diameter), maximum follicular diameters, rate of follicular growth per day, number of ovulation follicles, number of unovulated follicles and maximum diameter of CLs. It was found that the heifers treated with a 50 mg dose of FSHp had poor ovarian responses in this study. In contrast, when treated with a 150 mg dose of FSHp (Group 4), heifers showed high ovarian responses compared with the other groups.

The plasma levels of the FSH in Group 1 increased within 6 h after each injection of FSHp, and the relatively high levels of the hormone following each injection steadily decreased and sharply increased again after each injection (Fig. 1). The levels of FSH (1–1.8 ng/ml), however, were maintained in the circulation over the period of 6 to 48 h after the first injection. Moreover, the levels of FSH in the circulation in Group 1 were significantly higher than those of Groups 2 and 3 but lower than those in Group 4 (Fig. 1).

In Groups 2, 3 and 4, plasma levels of FSH sharply increased, peaked at 12 h after injection and gradually decreased thereafter (Fig. 1). In Group 4, the highest concentrations were maintained in the range of 0.7–2.2 ng/ml over a period of 6 to 66 h after injection FSHp in all groups ($P < 0.05$) (Fig. 1). Moreover, the maximum concentration range and maintenance period for FSH in the circulation after a single injection in Group 2 were lower than those in Groups 1, 3 and 4 (Fig. 1).

The plasma concentrations of progesterone (P4) in heifers of all the groups dramatically decreased to significantly lower levels by 18 h after the PGF_{2 α} injection; they continued to decrease till 96 h after the initiation of FSHp treatment compared with the P4 levels at 0 to 60 h after the initiation of FSHp treatment. Moreover, the heifers showed estrous behavior after the PGF_{2 α} injection in all groups. Meanwhile, during the same period as the P4 decrease, heifers

showing estrus showed sharp increases in the concentrations of plasma oestradiol-17 β (E2) at 54 and 60 h after injection of FSHp. These levels further increased until 96 h after injection of FSHp (Fig. 1).

The present study investigated the efficacy of superstimulation of follicular growth by a single i.m. injection of FSHp in Thai native heifers. Previous studies have reported that SOV treatment in Holstein cows [7, 8] and Japanese Black cows [9] by a single i.m. injection of 30 mg FSH dissolved in 30% PVP (wt/vol) (FSHp) produced a similar number of ova/embryos and transferable embryos as obtained by using the declining FSH dose method of administration. In addition, they also reported that a single i.m. injection of FSHp was capable of inducing an SOV response by maintaining a high plasma FSH concentration in order to enable the recovery of a sufficient number of embryos for transplantation. Relatively few studies have used a single i.m. administration of FSHp for ovarian stimulation prior to OPU. Ooe *et al.* [10] gave 20 mg FSHp as a single i.m. treatment prior to OPU in cyclic and pregnant Holstein cows and reported satisfactory follicular responses and oocyte recovery. Furthermore, Chaubal *et al.* [4] investigated the efficacy of a single i.m. injection of 200 mg FSHp for superstimulation of follicular growth treatment compared with multiple administrations in cyclic Angus-cross cows with respect to the number of oocytes recovered by the OPU technique. They reported no differences in the oocytes recovery rates between the groups receiving FSH in multiple administrations and those receiving a single i.m. administration. Consequently, FSHp administration as a single dose was adequate for ovarian stimulation in cyclic Angus-cross cows.

The present study, the Thai native heifers treated with a single 150 mg dose of FSHp showed high ovarian responses and the highest levels of plasma FSH (0.7–2.2 ng/ml) for a long period compared with other doses of FSHp. As a result, induction of follicular growth in Thai native heifers by a single injection of 150 mg FSHp seems to be most efficient in comparison with induction by the conventional multiple and single-dose injection methods using a total dose of 100 mg. However, this group showed a significantly higher number of unovulated follicles than the other groups ($P < 0.05$). This indicates that a single dose injection of 150 mg FSHp seems to induce an overmedicated state of SOV. It was previously found that although the number of total ova/embryos increased, the number of fertilized and transferable embryos decreased with a high dose of refined

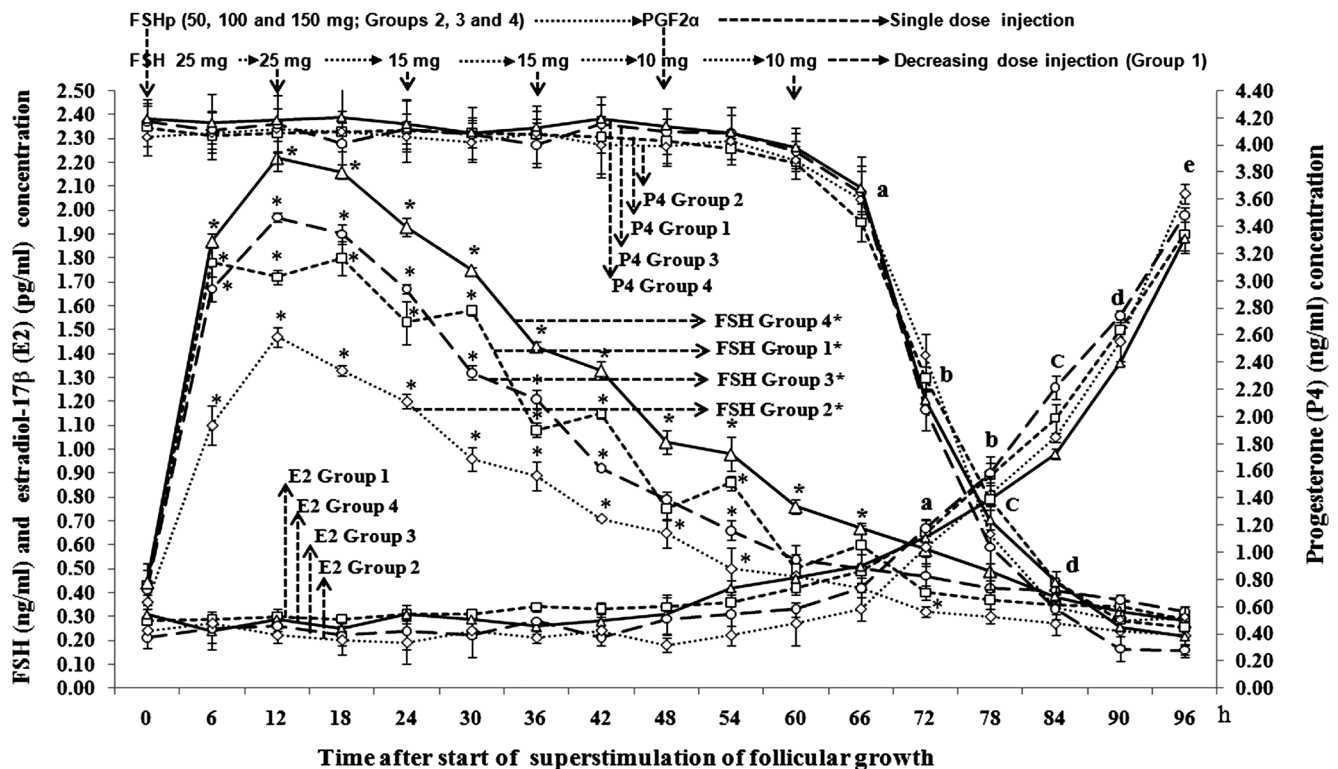


Fig. 1. Plasma progesterone (P4), estradiol-17 β (E2) and follicle stimulating hormone (FSH) concentrations (mean \pm SEM, $n = 4$ heifers) before exogenous FSHp (FSH dissolved in 30% PVP [wt/vol]) administration (0 h) up to 96 h after FSHp administration. Group 1 heifers were given multiple i.m. injections of FSH every 12 h for 3 days at decreasing doses, for a total of 100 mg, and served as controls. Groups 2, 3 and 4 heifers were given a single i.m. injection of FSHp at 50, 100 or 150 mg, respectively. Asterisks (*) indicate significant differences in concentrations of FSH during 0 h to 96 h after the initiation of FSHp injection among groups ($P < 0.05$). Superscripts (a,b,c,d,e) indicate significant differences in concentrations of P4 and E2 at the time of observation during 96 h after the initiation of FSHp injection among groups ($P < 0.05$); all the groups showed such similar concentrations of the hormones at the same times after initiation of the FSHp injections that the same superscripts (a,b,c,d,e) were used to indicate significant differences in concentrations of P4 and E2 at the time of observation in each group during the 96-h period after initiation of the FSHp injections.

follicle stimulating hormone (FSH-R) in cows [8]. Furthermore, when the dose of FSH-R was further increased, an adverse relationship between the number of ovulations and FSH-R dose was reported, with an actual and significant decrease in the numbers of ovulated and unovulated follicles [8]. Moreover, the SOV in cows administered various doses of pregnant mare serum gonadotrophin (PMSG) [12], human menopausal gonadotrophin (HMG) [13] and porcine follicle stimulating hormone (pFSH) [14] showed that a high level of follicle stimulation usually produced poor quality embryos and an increase in the number of unfertilized ova. The reason for the significant increase in unovulated follicle rate after treatment with a high level of FSH is unclear. However, it has been reported that administration of exogenous gonadotropin led to premature activation and degeneration of follicular oocytes; some of the activated oocytes were suggested to be retained in luteinized follicles, while others were ovulated as aged or degenerated ova [15].

In the present study, the number of unovulated follicles was lower in Group 3 than in Group 4. Meanwhile, the concentration of FSH in the circulation in Group 3 was maintained in the range of 0.5–2.0 ng/ml with a maintenance period of 6 to 66 h after a single injection of 100 mg/ml exogenous FSHp, and it is convincing that

the maintained concentrations of FSH in the circulation after a single injection of 100 mg/ml exogenous FSHp are suitable for inducing follicular development in Thai native heifers. In a previous study, Amporn and Vongpralub [5] and Wachchakool [16] investigated effects of FSH treatment on SOV in Thai native cattle, and they also reported that 100 mg was an optimal dose in superstimulation of follicular growth by decreasing doses of i.m. injections. Furthermore, Vongpralub *et al.* [6] reported that 100 and 200 mg of FSH for i.m. injection by decreasing doses were optimum doses in superstimulation of follicular growth in Thai native heifers and cows.

The results of the present study show that in all groups of heifers that were given a single i.m. injection of FSHp, plasma levels of FSH sharply increased, peaked at 12 h after injection and gradually decreased thereafter. Interestingly, heifers in Group 3 that were given 100 mg of FSHp by single-dose injection showed plasma concentrations of FSH that ranged from 0.5 to 2.0 ng/ml and were maintained from 6 to 66 h after injection. These results were similar to the reports for SOV in Holstein heifers [6] and cows [17] given a single-dose injection of 30 mg FSHp, and they reported that after a single injection of FSHp, the plasma levels of FSH gradually increased, peaked at 12 h after injection and then decreased gradu-

ally. Also, the highest concentration ranges of FSH in circulation were maintained at 40–80 ng/ml from 3 to 60 h after single FSHp injections. Therefore, the findings of this study and previous studies suggested that PVP is a suitable solvent for the absorption of FSH (FSHp) and that a single i.m. injection of FSHp solution can induce follicular development in cattle.

In conclusion, the results of the present study revealed that superstimulation of follicular growth in Thai native heifers by a single i.m. administration of FSH dissolved in 30% PVP (wt/vol) (FSHp) could induce follicular growth after treatment when compared with the conventional method of twice daily administrations over a 3-day period. The most effective dose of FSHp for Thai native heifers appears to be 100 mg. However, the present study did not investigate the quality of embryos or oocytes obtained by a single-dose injection of FSH dissolved in PVP. Therefore, further experiments on the quality of embryos or oocytes are warranted to investigate if this novel method of pFSH treatment is effective for successful SOV or OPU in Thai native cattle.

Materials and Methods

Animals and treatments

The research protocol was approved by the Animal Ethical Committee of Khon Kaen University, Thailand. Before FSH treatments, the estrus cycles of 16 Thai native heifers (2.13 ± 0.14 years old and 187.52 ± 1.82 kg; mean \pm SEM) were synchronized by injecting 500 μ g PGF_{2 α} (Cloprostenol, Estrumate; Coopers, Berkhamsted, England) twice at an interval of 12 days. Follicular growth in heifers was stimulated by injection of pFSH between days 9 and 12 of the estrous cycle (day of estrus = day 0) [11].

To prepare follicle stimulating hormone dissolved in PVP (FSHp), firstly 30 g of PVP (PVP-40, molecular weight 40,000; Sigma-Aldrich Chemical, St. Louis, MO, USA) was dissolved in 100 ml of distilled water to produce a 30% (wt/vol) PVP solution and sterilized by autoclaving. Then it was divided into aliquots of 10 ml each and stored at 4 C. Just before use, FSH (Folltropin®-V; Bioniche Animal Health, Belleville, ON, Canada) was dissolved at 20 mg/ml in diluent (Folltropin®-V Diluent; Bacteriostatic Sodium Chloride Injection, Bioniche Animal Health) and mixed well with 10 ml of 30% PVP solution to create pFSH [11].

There were 4 treatment groups in this study. Group 1 heifers (n=4) were given multiple i.m. injections of pFSH every 12 h for 3 days at decreasing doses, for a total of 100 mg, and served as the controls. Groups 2, 3 and 4 heifers (n=4 in each group) were given a single i.m. injection of 50, 100 or 150 mg FSHp, respectively. All animals received a single injection of 750 μ g PGF_{2 α} 48 h after the initiation of pFSH treatment.

Ultrasonography evaluations

Ovarian follicular activity was monitored daily by real-time transrectal ultrasonography (Honda®HS-2000, Honda Electronics, Toyohashi, Aichi, Japan) equipped with a 7.5-MHz probe from the day of initiation of pFSH treatment until the occurrence of estrus. The sonograms from ultrasonographic scanning of both ovaries were recorded in the ultrasound machine at examination; the diameters and locations of all follicles (≥ 2 mm in diameter) were recorded on

a sketch of each ovary and analyzed retrospectively. The diameters of the dominant follicles and largest subordinate follicles were evaluated, and rates of follicular growth per day were calculated. Representative sonograms were created by digitizing the ultrasound sequence using Adobe Premiere Pro 1.5 (Adobe Systems, Amsterdam, The Netherlands), and the regions of interest were measured on the sonograms in the JPEG format with the AnalySIS 3.1 software program (Olympus Soft Imaging Solution, Munster, Germany). The number of large follicles (≥ 5 mm in diameter), maximum follicular diameter, rate of follicular growth per day, number of ovulation follicles, number of unovulated follicles and maximum diameters of CLs were recorded.

Hormone analyses

Blood samples were collected from the animals by coccygeal artery puncture, using 15 ml vacutainer tubes containing heparin. Blood collections were done at 0 h just before the start of treatment, and 6, 12, 24, 36, 48, 60, 72, 84 and 96 h after the initiation of the treatment. The samples were centrifuged at 2,000 rpm for 20 min, and the plasma obtained was identified and stored at –20 C until measurements of hormone concentrations. The analyses were performed by the radioimmunoassay (RIA) technique for determination of FSH, P4 and E2 in the radioimmunoassay laboratory, Department of Radiology, Faculty of Medicine, Khon Kaen University, Thailand.

Plasma concentrations of porcine FSH (pFSH) were measured by heterologous radioimmunoassay, as previously described [7]. Anti-pFSH rabbit serum and purified pFSH were used for iodination; reference standards were purchased from UCB-Bioproducts SA (Braine-l'Alleud, Belgium). The analytical sensitivity of this assay was 0.049 ng/ml. The intra- and interassay coefficients of variation were 5.9 and 7.4%.

Plasma concentrations of E2 and P4 were determined, as described previously [18], using antisera to E2 (GDN 244 [19]) and P4 (GDN 337 [20]). The analytical sensitivity of this assay was 0.19 pg/ml for E2 and 0.58 ng/ml for P4. The intra- and interassay coefficients of variation were 6.2 and 7.4% for E2, respectively, and 3.9 and 9.3% for P4, respectively.

Statistical analysis

An analysis of variance was carried out, and Duncan's multiple range tests [21], the student *t*-test and paired *t*-tests were used to determine the significance of the difference means ($P < 0.05$).

Acknowledgments

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Food and Functional Food Research Cluster of Khon Kaen University.

References

1. Tribulo A, Rogan D, Tribulo H, Tribulo R, Alasino RV, Beltramo D, Bianco I, Maplettofe RJ, Bo GA. Superstimulation of ovarian follicular development in beef cattle with a single intramuscular injection of Folltropin-V. *Anim Reprod Sci* 2011; **129**: 7–13. [Medline] [CrossRef]

2. **Kimura K, Hirako M, Iwata H, Aoki M, Kawaguchi M, Seki M.** Successful superovulation of cattle by a single administration of FSH in aluminum hydroxide gel. *Theriogenology* 2007; **68**: 633–639. [Medline] [CrossRef]
3. **Bo GA, Hockley DK, Nasser LF, Mapletoft RJ.** Superovulatory response to a single subcutaneous injection of Follitropin-V in beef cattle. *Theriogenology* 1994; **42**: 963–975. [Medline] [CrossRef]
4. **Chaubal SA, Ferre LB, Molina JA, Faber DC, Bols PEJ, Rezamand P, Tian X, Yang X.** Hormonal treatments for increasing the oocyte and embryo production in an OPU-IVP system. *Theriogenology* 2007; **67**: 719–728. [Medline] [CrossRef]
5. **Amporn C, Vongpralub T.** Effects of follicle stimulating hormone levels on ovulatory response and embryos quality in Thai native cattle. In: 4th Animal Science conference. Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand, 2008; pp: 144–147 (In Thai).
6. **Vongpralub T, Sanchaisuriya P, Paopha P, Sangkaew A, Wongshi M, Sontichai M.** Research report: The deep-freeze preservation of semen and embryo of Thai native cattle. Research grants from annual government statement of expenditure in 1999, General grants category. Khon Kaen: Khon Kaen University; 2002 (In Thai).
7. **Takedomi T, Aoyagi Y, Konishi M, Kishi H, Taya K, Watanabe G, Sasamoto S.** Superovulation in Holstein heifers by a single injection of porcine FSH dissolved in polyvinylpyrrolidone. *Theriogenology* 1994; **39**: 327–348. [CrossRef]
8. **Yamamoto M, Kawaguchi M, Ooe M, Takagi M, Suzuki T.** Dose response to a single intramuscular injection of FSH dissolved in polyvinylpyrrolidone for superovulation in cows. *J Reprod Dev* 1995; **41**: 93–96. [CrossRef]
9. **Yamamoto M, Ooe M, Kawaguchi M, Suzuki T.** Superovulation in the cow with a single intramuscular injection of FSH dissolved in polyvinylpyrrolidone. *Theriogenology* 1994; **41**: 747–755. [Medline] [CrossRef]
10. **Ooe M, Rajamahendran R, Boediono A, Suzuki T.** Ultrasound guided follicle aspiration and IVF in dairy cows treated with FSH after removal of the estrous cycle. *J Vet Med Sci* 1997; **59**: 371–376. [Medline] [CrossRef]
11. **Yamamoto M, Suzuki T, Ooe M, Takagi M.** Superovulation in Beef cows and heifers with a single injection of FSH dissolved in polyvinylpyrrolidone. *J Reprod Dev* 1993; **39**: 353–356. [CrossRef]
12. **Booth WD, Newcomb R, Strange H, Rowson LEA, Sacher HB.** Plasma oestrogen and progesterone in relation to superovulation and egg recovery in the cow. *Vet Rec* 1975; **97**: 366–369. [Medline] [CrossRef]
13. **McGowan MR, Braitwaite M, Jochle W.** Superovulation of beef heifers with Perginal (HMG): A dose response trial. *Theriogenology* 1985; **24**: 173–184. [Medline] [CrossRef]
14. **Elsden RP, Kessler RM.** Superovulation of Nelore cows and Heifers. *Theriogenology* 1983; **19**: Abstract 127.
15. **Moor RM, Kruij Th AM, Green D.** Intraovarian control of folliculogenesis: Limits to superovulation? *Theriogenology* 1984; **21**: 103–116. [CrossRef]
16. **Wachchakool W.** Superovulation in Thai native cattle by follicle stimulating hormone and pregnant mare serum gonadotrophin. Master of Science Thesis in Animal Science, Graduate School. Khon Kaen: Khon Kaen University; 2001 (In Thai).
17. **Lovie M, Garcia A, Hackett A, Mapletft RJ.** The effect of dose schedule and route of administration on superovulation on superovulatory response to follitropin in Holstein cows. *Theriogenology* 1994; **41**: 241. [CrossRef]
18. **Taya K, Watanabe G, Sasamoto S.** Radioimmunoassay for progesterone, testosterone and estradiol-17 β using using ¹²⁵I-iodohistamine radioligands. *J Anim Reprod* 1985; **31**: 186–197. [CrossRef]
19. **Korenman SG, Stevens RH, Carpenter LA, Robb M, Niswender GD, Sherman BD.** Estradiol radioimmunoassay without chromatography: procedure, validation and normal values. *J Clin Endocrinol Metab* 1974; **38**: 718–720. [Medline] [CrossRef]
20. **Gibori G, Antczak E, Rothchild I.** The role of estrogen in regulation of ruteal progesterone secretion in the rat after day 12 of pregnancy. *Endocrinology* 1977; **100**: 1483–1495. [Medline] [CrossRef]
21. **Stell RGD, Torrie JH.** Principles and Procedures of Statistics: A Biometrical Approach, 2nd ed. New York: McGraw-Hill Book Coy. Inc., 1980.