Research Report

FERTILIZATION RATE AFTER DEEP INTRA UTERINE INSEMINATION IN PIG

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4. Abstract/ Research summary

The objectives of the present study were to investigate fertilization rate and number of embryo on each side of the reproductive tract after inseminated multiparous sows using deep intra uterine insemination (DIUI) technique with fresh semen. Eight crossbred multiparous sows were used in the experiment. The sows were examined for the onset of standing oestrus every 6 h by using a back pressure test in the presence of a mature boar. Transrectal ultrasonography was performed every 4 h using a 5 MHz probe to examine the time when ovulation took place in all sows. During the second oestrus after weaning, the sows were inseminated once at about 6-8 h before expected ovulation using diluted fresh semen from a proven sire. The insemination was performed using DIUI technique. The long flexible catheter (1.8 m) was inserted through the conventional artificial insemination (AI) catheter and was moved forward along one uterine horn until its total length. The diluted fresh semen with $150x10^6$ motile sperm in 5.0 mL Beltsville thawing solution (BTS) was deposited at the proximal third in one side of the uterine horn (unknown side). Subsequently, a warm BTS 2.5 mL was used to flush the semen into the uterine horn after insemination. All sows (n=8) were generally anesthetized at about 61.1±12 h after insemination (range 48-72 h). The embryos were flushed from the oviduct and the proximal part of the uterine horn. The number of embryos and number of ovulation on the left and right sides of the reproductive tract within animal were compared using pair *t*-test. It was found that fertilization was found in 5 out of 8 sows (62.5%) after DIUI. On average, 11.2±2.2 embryos/sows were recovered from both sides of the reproductive tracts. The number of ovulation per sows was 16.4±2.6 ova. The number of ovulation on the left and the right side of the ovaries did not differ significantly (8.5 vs 7.9; P=0.75). Of the 5 pregnant sows, 57 embryos were recovered. Of these embryos, 3 embryos developed to 8-cell stages (5.3%) and 54 embryos developed to 4-cell stages (94.7%). Number of embryos recovered from the left and the right side of the reproductive tracts were not significantly differences (left-right =+4.2, P=0.20). The overall recovery rate of the oocytes and embryos was 66.4% (87/131). Fertilization rate in all pregnant sows was 100% (all ovum were fertilized). It could be concluded that using DIUI technique in pig with $150x10^6$ spermatozoa, fertilization took place in both side of the oviducts.

Keywords: pig, reproduction, artificial insemination, embryo

5. Research background

Artificial insemination (AI) in pig is nowadays widely used in the pig industry all over the world. Under field conditions, $2\text{-}5x10^9$ motile spermatozoa in 80-100 mL of volume are inseminated to the sow intra-cervically for 2-4 times during the standing oestrus. Recently, deep intra uterine insemination (DIUI) using a special designed catheter, length 180 cm outer diameter 4 mm and working channel 1.8 mm, has been developed (Martinez et al., 2001; Vazquez et al., 2005). The catheter could be inserted through the uterine horn and deposited semen in one horn at the proximal third of the uterine horn, closed to the sperm reservoir. Earlier studies have shown that the flexible catheter could be passed through the cervix completely in 90-95% of multiparous sows (parities 2-6; n=147) within about 4 min/insemination (Martinez et al. 2001, 2002). Using this technique a 20-fold reduction in the number of spermatozoa could be used without any significant effect on farrowing rate (FR) and litter size (Martinez et al., 2002). Martinez et al. (2002) demonstrated that FR and number of total piglets born/litter (TB) after DIUI with $150x10^6$ spermatozoa/dose in 5 mL volume compared to

conventional AI with 3,000 x 10^6 spermatozoa/dose in 100 mL volume did not differ significantly (FR 82.9 versus 83% and TB 9.7 versus 9.9 piglets/litter, respectively).

DIUI technique have also been applied for some new sperm technology e.g., frozen-thaw semen and sex sorted sperm and maximizing the used superior boar sperm by using a low sperm number per dose of insemination (Roca et al., 2003; Vazquez et al., 2005). Roca et al. (2003) inseminated 49 hormonally treated weaned sows once with frozen-thawed sperm 1000x10^6 spermatozoa/dose in 5 mL using DIUI technique resulted in 77.5% FR and 9.3 piglets/litter, which was not differ significantly from frozen-thawed sperm 6000x10^6 spermatozoa/dose in 100 mL inseminated using conventional AI technique (75.8% FR and 9.6 piglets/litter, n=33). In normal weaned sows inseminated twice at 30 and 42 h after onset of oestrus with DIUI technique during oestrus, frozen-thaw semen (1,000x10^6 spermatozoa/dose) resulted in a lower FR than fresh semen (150x10^6 spermatozoa/dose) (70.0 versus 84.2%). Vazquez et al. (2003) demonstrated that hormonally treated weaned sows (n=45) inseminated once at 38 h after HCG treatment with 140x10^6 flowcytometry sorted spermatozoa using DIUI technique resulted in 46.6% FR and 8.2 piglets/litter, while the sows inseminated with fresh semen (n=49) using the same technique and same number of spermatozoa resulted in 80.9% and 9.5 piglets/litter. Up to date, the highest speed to sorted into X and Y population is about 10-15x10^6 spermatozoa/h (Johnson and Welch, 1999), which is too slow for routine application in pig. In general, spermatozoa after frozen-thawed or flowcytometry sex sorting have a shorter life span than spermatozoa from fresh semen. Therefore, these spermatozoa need to be deposited close to the site of fertilization to avoid premature death that might occur during transport from cervix to the sperm reservoir.

For conventional AI in pig, 25% of the spermatozoa inseminated were loss due to semen backflow within a few hours after insemination (Steverink et al., 1998). The rest of the spermatozoa were transport through the uterine horn before reaching the sperm reservoir in the caudal part of the oviducts. It was found that less than 1% of sperm that were inseminated was recovered in both sides of the sperm reservoir (Mburu et al., 1996). Most of the spermatozoa lost due to uterine phagocytosis by polymorphonuclear leucocyte (Rozeboom et al., 1998; Kaeoket et al., 2003). DIUI technique was developed to avoid the loss of spermatozoa and ensure optimal fertilization (Martinez et al., 2001). Similar technique have already been reported in cattle (Seidel et al. 1997; Hunter, 2003; Verberckmoes et al., 2004), horses (Morris et al., 2000), dog (Tsutsiu et al., 1989) and cat (Tsutsui et al., 2000) and goat (Sohnrey and Holtz, 2005).

Up to date, data concerning sperm transport and fertilization after DIUI with a small volume of semen in pig are still limited. Martinez et al. (2002) demonstrated that the embryos were found in both side of the tip of the uterine horn at 2 days after DIUI in 5 sows. On the other hand, our previous finding found that the spermatozoa deposited in only one side of the uterine horn at 24 h after DIUI (Tummaruk et al., 2005). The fertilization process after DIUI technique is therefore needed to be investigated further.

6. Objectives

The objectives of the present study were to investigate fertilization rate and number of embryo on each side of the reproductive tract after inseminated multiparous sows using DIUI technique with fresh semen.

7. Research methodology

Experimental research

8. Time period

1st October 2004- 30th September 2005

9. Materials and methods

Animals

Eight crossbred (Landrace x Yorkshire) multiparous sows were used in the experiment. On the day of weaning, they were brought from commercial farms to the Department of Obstetrics, Gynaecology

and Reproduction, Faculty of Veterinary Science, Chulalongkorn University and were allocated to individual pens adjacent to adult boars. The sows were fed 3 kg per day (twice a day) with a commercial feed (Starfeed176® BP Feed Co. Ltd, Saraburi, Thailand) containing protein 15%, fat 2% and fiber 10%. Water was provided ad libitum. The sows were detected for pro-oestrus twice a day (am/pm) after weaning.

Detection of oestrus and ovulation

After the sows showed signs of pro-oestrus, the sows were examined for the onset of standing oestrus every 6 h by using a back pressure test in the presence of a mature boar. Onset of oestrus was defined as being 3 h before the onset of the standing response. The end of oestrus was defined as 3 h after the last standing response. Transrectal ultrasonography was performed every 4 h using a 5 MHz probe to examine the time when ovulation took place in all sows. Ovulation time was 2 h after the last detection of follicles on the ovaries. Previous study has shown that the interval from onset of oestrus to ovulation within animal during the first two cycles after weaning was not significantly difference and the repeated ultrasonographic examination can predict the time of ovulation during the subsequent oestrus (Mburu et al., 1995). In the present study, the time of ovulation in each individual sows were used to estimate the time of insemination during the subsequence oestrus cycle. The ultrasonographic examination was not performed after insemination, to not disturb the sows and the process of sperm and/or oocyte transport.

Collection and dilution of semen

The semen was collected from an adult proven boar by the gloved-hand method. Semen was examined for quality before further processing i.e., motility, concentration and morphology. Semen with motility ≥70%, concentration ≥150 spermatozoa/mL and normal sperm ≥85% was diluted, using Beltsville thawing solution (BTS) diluent (Pursel and Johnson, 1976) at 35 °C. The sperm dose contained $150x10^6$ spermatozoa in 5 mL. The diluted semen was used immediately or kept at 18 °C for no longer than 2 days before insemination. The refrigerated dilute semen was warmed in water bath at 35 °C for 15 min and was checked for motility before being used. For all inseminations, the pre-insemination diluted semen had to have a motility ≥60%.

Insemination

During the second oestrus after weaning, the sows were inseminated once at about 6-8 h before expected ovulation using diluted fresh semen from a proven sire with an individual motility of at least 60%. The time of ovulation (determined by ultrasonography) during the first oestrus was used to determine the timing of insemination.

The insemination was performed using DIUI technique, which have been described by Martinez et al (2001). Briefly, the oestrous sows were inseminated within the gestation crates. After cleaning the perineal area of the sows, a commercial AI catheter (Goldenpig®, Minitub, Germany) was inserted through the vagina into the cervix. The long flexible catheter (1.8 m) was inserted through the conventional AI catheter. The long catheter was moved forward along one uterine horn (unknown side) until its total length. The diluted fresh semen with $150x10^6$ motile sperm in 5.0 mL was deposited at the proximal third in one side of the uterine horn. Subsequently, a warm BTS 2.5 mL was used to flush the semen into the uterine horn after insemination.

Embryo collection

All sows (n=8) were generally anesthetized at about 61.1±12 h after insemination (range 48-72 h). The sows were sedate with 2 mg/kg azaperone (Stressnil®) intra muscularly. Thiopental sodium (10 mg/kg) was used intravenously for general anesthesia. The sows were operated at caudal midline incision about 10-20 cm length with ventral recumbency position. Left and right uterine horns and the ovaries were approached. Number of corpora lutea on each side of the ovary was counted (Fig 1a). A 1 cm incision was made at about 20 cm below the utero-tubal junction (UTJ). A Foley catheter were inserted through the incision and moved forward to the tip of the horn and then the balloon was made at the tip of the catheter (Fig. 1b). A polyethylene catheter (outer diameter 2.42 mm) was inserted from the end of the oviduct (infundibulum) and was fixed with the oviduct (Fig 1c). A 100 mL phosphate buffer solution (PBS) was filled into the tip of the oviduct through the uterine horn (Fig. 1c). The PBS solution was forced to release into the Foley catheter where flask bottle were used to collect all the flushed solution (Fig. 1d). Using this technique, the embryos were flushed from the oviduct and the

proximal part of the uterine horn (Fig. 2). The number of embryos and number of ovulation on the left and right sides of the reproductive tract within animal were compared. Recovery rate were the number of embryo divided by number of corpora lutea and then multiply by one hundred. Fertilization rate was defined as number of embryo divided by total number of embryo and unfertilized ova found and then multiply by one hundred. Number of embryos and fertilization rate were compared between left and right side of the uterine horn within animal.

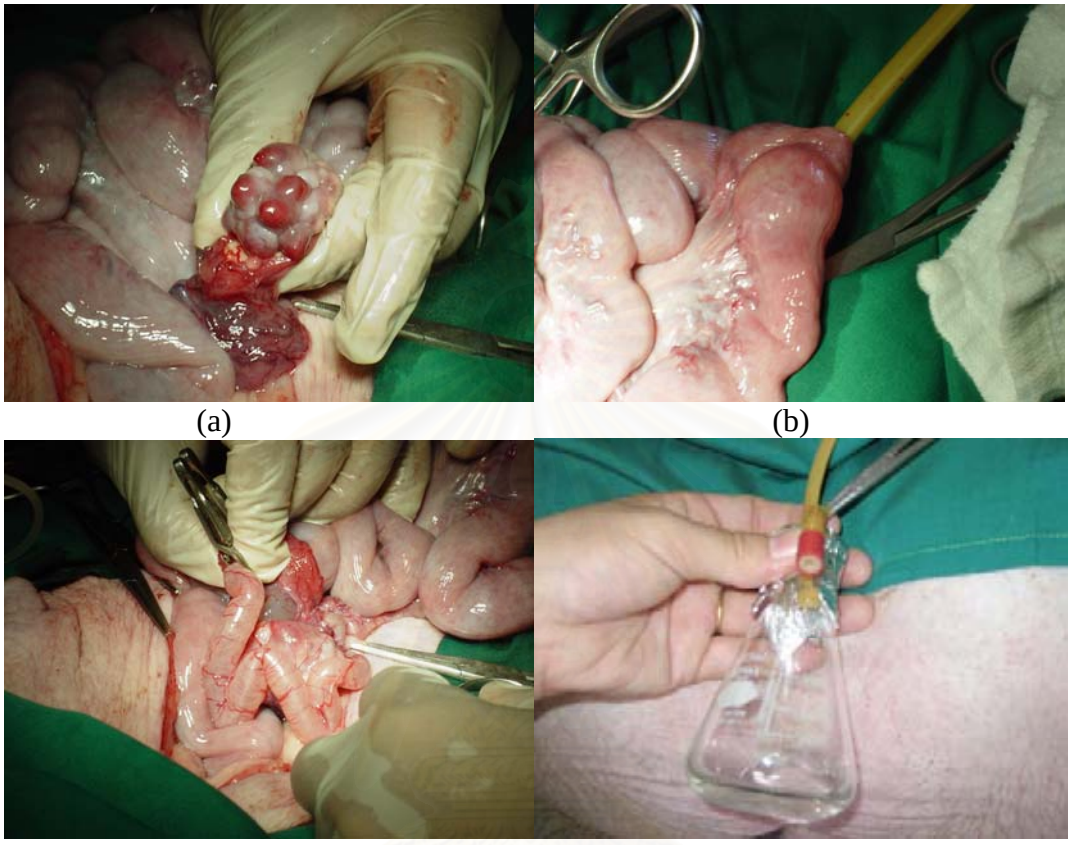

Fig. 1 Embryo collection procedure (a.) approaching the ovaries and uterus and count number corpora lutea (b.) insert Foley catheter and made a balloon in the proximal part of the uterus (c.) flushing embryos from oviduct (d.) using flask bottle to collect all flushing solution.

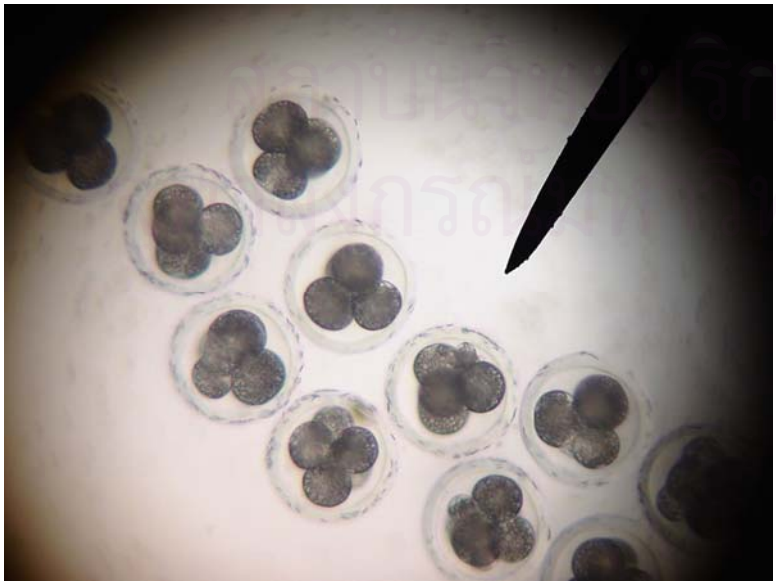

Fig 2. Embryos at 4 cell stage after collection.

Statistical analyses

The statistical analyses were carried out using SAS version 9.0 (SAS, 1996). The number of ovulation in all sows (n=8), number of embryo in the pregnant sows (n=5) and number of non-fertilized egg in non-pregnant sows (n=3) between left and right side of the uterine horns were compared using pair *t*-test. The differences with $P<0.05$ were regarded as statistical significance.

10. Results

On average, the sows were inseminated at 30.9±7.3 h after the onset of standing oestrus and the surgical collection of embryos was performed at 61.1±12 h after insemination. Fertilization was found in 5 out of 8 sows (62.5%) after DIUI (Table 1). On average, 11.2±2.2 embryos/sows were recovered from both sides of the reproductive tracts. The overall recovery rate of the oocytes and embryos was 66.4% (87/131). On average, the number of ovulation per sows was 16.4±2.6 ova (range 14-22 CL). The number of ovulation on the left and the right side of the ovaries did not differ significantly (8.5 vs 7.9; $P=0.75$).

For the pregnant sows, the embryos were found in both sides of the reproductive tracts in all sows (5/5 sows) (Table 1). Of the 5 pregnant sows, 57 embryos were recovered. Of these embryos, 3 embryos developed to 8-cell stages (5.3%) and 54 embryos developed to 4-cell stages (94.7%). Number of embryos recovered from the left and the right side of the reproductive tracts were not significantly differences (left-right =+4.2, $P=0.20$) (Table 1). Number of non-fertilized eggs recovered from the left and the right side of the reproductive tracts were not significantly differences (left-right =-0.6, $P=0.66$) (Table 1). Fertilization rate in all pregnant sows was 100% (all ovum were fertilized).

Table 1 Descriptive statistics (mean±SD) on number of corpus luteum (CL), number of embryos and unfertilized ova on the left and right sides of the oviducts at 61.1±12 h after deep intra-uterine insemination (DIUI) in pregnant and non-pregnant sows.

Parameters	Pregnant sows (n=5)		Non-pregnant sows (n=3)	
	Left	Right	Left	Right
No. of CL	9.4±3.6	7.4±3.6	7.0±1.0	8.7±1.5
No. of embryos	7.8±3.9	3.6±2.5	0	0
No. of unfertilized ova	0	0	4.7±2.3	5.3±2.3
Recovery rate (%)	39/47 (82.9%)	18/37 (48.6%)	14/21 (66.7%)	16/26 (61.5%)

11. Discussion & Application

The present study demonstrated that using DIUI technique with a low number of spermatozoa, the embryos were found in both side of the uterine horn of the pregnant sows. This finding is in agreement with Martinez et al. (2002). However, our previous study found that the spermatozoa after DIUI deposited in only one side of the oviduct during the first 24 h after insemination (Tummaruk et al., 2005). These findings indicated that the spermatozoa might be transported from one to another horn of the uterus sometime between 24-48 h after insemination. We believed that the spermatozoa were distributed from only one side of the sperm reservoir prior to the time of fertilization. The mechanism for the sperm transport and fertilization may need further investigation in sows. However, trans-peritoneal migration of the spermatozoa has been reported in heifer (Larsson, 1986).

In the present study, five out of eight got pregnant and 11.2 embryos/sows were recovered after DIUI with a low number of spermatozoa, indicating about 62.5% pregnancy rate and 11.2 piglets within the uterus. In other studies where the same technique has been applied, FR varied between 82-86% and means total number of piglets born per litter varied between 9.7-10.0 piglets/litter (Martinez et al., 2002; Roca et al., 2003). Roca et al. (2003) found that the hormonal treated (eCG/HCG) weaned sows (n=29) inseminated once with $150x10^6$ spermatozoa by DIUI technique with fresh semen had 82.7% FR and 9.96 piglets born per litter. Spontaneously weaned sows (n=38) inseminated twice with $150x10^6$ sermatozoa by DIUI technique with fresh semen resulted in 84.2% FR and 9.88 piglets born per litter (Roca et al. 2003). Martinez et al. (2002) found that DIUI in one side of the uterine horn with $150x10^6$ spermatozoa resulted in 86.3% pregnancy rate, 82.9% FR and 9.7% total piglets born per litter (n=117). Reduce number of spermatozoa below $25x10^6$ spermatozoa/dose resulted in a significantly decrease in FR (Martinez et al., 2002).

In the present study, both 4 and 8 cells embryos were found. This could be explained by the fact that in pig, the duration of ovulation in spontaneous ovulating is about 1-3 h (Soede et al. 1992). Pope et al. (1990) showed that 70% of follicles ovulated during a short period of time while the remaining follicles ovulated over a protracted period. Oocyte of follicles predicted to ovulated first became the more develop embryos, while oocyte from later ovulating follicle became the less develop embryo. Ova maybe transport to the fertilization site at different times, and then become fertilize at different time. Unlike the mare, both fertilize and unfertilized ova of the sow were transport via the oviduct and enter the uterus in the second day after ovulation (Mwanza et al., 2002). Mwanza et al. (2002) found that there is no difference in the transportation of fertilize and unfertilized ova in the reproductive tract of pigs.

Martinez et al. (2006) demonstrated that total number of piglets born per litter after DIUI in spontaneous oestrus sows significantly smaller than conventional AI (9.8 versus 10.9), although the FR was not significantly difference. In addition, when embryos on day sixth after oestrus was observed, it was found that partial fertilization was higher in the DIUI sows (35%) compared with the conventional AI sows (5%). The fertilization after DIUI took place in both uterine horns in about 80% of the sows, while unilateral fertilization was found in about 20% of sows (Martinez et al., 2006). In the present study, the fertilization in sows seems to be all or none phenomenon. Ovum from the five pregnant sows was fertilized all, while ovum from the three non-pregnant sows was not fertilized all. The partial and/or unilateral fertilization were not found in the present study. The reason might be due to that the sows in the present study were inseminated once at 6-8 h before ovulation, while in earlier study (Martinez et al. 2006), the sows were inseminated 3 times (12, 24 and 36 h after onset of oestrus) with regardless to the timing of ovulation. Variation on the timing of ovulation in spontaneous ovulation sows might cause partial and/or unilateral fertilization. In dog, a surgical unilateral insemination with sperm number $\geq 20x10^6$ spermatozoa in 0.1 mL resulted in bilateral fertilization, while insemination with sperm number $\leq 10x10^6$ spermatozoa in 0.1 mL resulted in unilateral fertilization (Tsutsui et al., 1989).

It could be concluded that using DIUI technique in pig with $150x10^6$ spermatozoa, fertilization took place in both side of the oviducts.

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13. Research output

Tummaruk, P., P. Sumransarp, S. Thongyu, W. Boontengcharn, S. Suksudprasert, W. Adulyanubap, J. Singlor & M. Techakumphu 2005. Fertilization takes place in both sides of the oviducts after deep intra uterine insemination in pig. Proc AHAT/BSAS international conference. November 14-18, 2005. Khon-kaen, Thailand. P. T109. (Oral presentation).

Fertilization takes place in both sides of the oviducts after deep intra uterine insemination in pig

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Summary

The present study demonstrated the fertilization on each side of the oviduct in 8 sows after applying a new technique for artificial insemination (AI) in pig, deep intra uterine insemination (DIUI), with a low number of spermatozoa per dose. It was revealed that embryos were found in both side of the oviducts in all pregnant sows (5/8 sows). The average number of embryos recovered at about 3 days after insemination was 11.2±2.2 per sows. It could be concluded that using DIUI technique with a low number of spermatozoa, fertilization took place in both side of the oviducts.

Keywords: pig, reproduction, artificial insemination, embryo

Introduction

AI in pig is nowadays widely used in the pig industry all over the world. Under field conditions, 2-5x10^9 motile spermatozoa in 80-100 mL of volume are inseminated to the sow for 2-4 times during the standing heat. Recently, DIUI has been developed using a special designed catheter (Vazquez et al., 2005). The catheter could be inserted through the uterine horn and deposited semen in one horn at the proximal third of the uterine horn, closed to the sperm reservoir. Using this technique a 20-fold reduction in the number of spermatozoa could be used without any significant effect on farrowing rate and litter size (Martinez et al., 2002). The technique is also applicable for some new sperm technology e.g., frozen-thaw semen and sex sorted sperm and maximizing the used superior boar sperm by using a low sperm number per dose of insemination (Vazquez et al., 2005). Investigations on sperm distribution and fertilization after DIUI technique need to be performed. The aims of the present study were to investigate number of embryo and fertilization rate on each side of the reproductive tract after DIUI with a low number of spermatozoa per dose.

Materials and Methods

Eight crossbred (LY) multiparous sows were used in the experiment. The sows were detected for standing oestrus twice a day (am/pm) after weaning. Transrectal ultrasonography was used for detection of ovulation. Sows returned to oestrus within 6 days after weaning and ovulated normally were included in the experiment. During the second oestrus after weaning, the sows were inseminated once at about 6-8 h before expected ovulation using diluted fresh semen from a proven sire with an individual motility of at least 70%. The sows were inseminated using DIUI technique with 0.15x10^9 motile sperm in 7.5 mL of volume. The spermatozoa were deposited at the proximal third in one side of the uterine horn (unknown side). The sows were generally anesthetized and the embryos were flushed from the oviduct and the proximal part of the uterine horn between 48-72 h after insemination. The number of embryos and number of ovulation on the left and right sides of the reproductive tract within animal were compared. The statistical analyses were carried out using SAS. The differences with $P<0.05$ were regarded as statistical significance.

Results and Discussion

On average, the sows were inseminated at 30.9±7.3 h after the onset of standing oestrus and the surgical collection of embryos was performed at 61.1±12 h after insemination. Fertilization was found in 5 out of 8 sows (62.5%) after DIUI (Table 1). On average, 11.2±2.2

embryos/sows were recovered from both sides of the reproductive tracts. The overall recovery rate of the oocytes and embryos was 66.4% (87/131). On average, the number of ovulation per sows was 16.4±2.6 ova (range 14-22 CL). The number of ovulation on the left and the right side of the ovaries did not differ significantly (8.5 vs 7.9; *P*=0.68). For the pregnant sows, the embryos were found in both sides of the reproductive tracts in all sows (5/5 sows) (Table 1). Of the 5 pregnant sows, 57 embryos were recovered. Of these embryos, 3 embryos developed to 8-cell stages (5.3%) and 54 embryos developed to 4-cell stages (94.7%). Regardless to the side of the insemination, number of embryos recovered from the left and the right side of the reproductive tracts were not significantly differences (7.8 vs 3.6, *P*=0.08) (Table 1). The present study demonstrated that using DIUI technique with a low number of spermatozoa, the embryos were found in both side of the uterine horn of the pregnant sows. This finding is in agreement with Martinez et al. (2002). Our previous study found that the spermatozoa after DIUI deposited in only one side of the oviduct during the first 24 h after insemination (Sumransap et al., 2004). These findings indicated that the spermatozoa might be transported from one to another horn of the uterus sometime between 24-48 h after insemination. We believed that the spermatozoa were distributed from only one side of the sperm reservoir prior to the time of fertilization. The mechanism for the sperm transport and fertilization may need further investigation in sows. However, trans-peritoneal migration of the spermatozoa has been reported in heifer (Larsson, 1986).

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Table 1. Descriptive statistics (mean±SD) on number of corpus luteum (CL), number of embryos and unfertilized ova on the left and right sides of the oviducts at 61.1±12 h after deep intra-uterine insemination (DIUI) in pregnant and non-pregnant sows

	Pregnant sows (n=5)		Non-pregnant sows (n=3)	
	Left	Right	Left	Right
No. of CL	9.4±3.6	7.4±3.6	7.0±1.0	8.7±1.5
No. of embryos (4-8 cells)	7.8±3.9	3.6±2.5	0	0
No. of unfertilized ova	0	0	4.7±2.3	5.3±2.3
Recovery rate (%)	39/47 (82.9%)	18/37 (48.6%)	14/21 (66.7%)	16/26 (61.5%)