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—Original Article—

# Factors Affecting the Fertility of Ewes after Intrauterine Insemination with Frozen-Thawed Semen During the Non-Breeding Season

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**Abstract.** In this study, two successive field trials were conducted during the non-breeding season to investigate various factors affecting on fertility of Suffolk ewes after intrauterine insemination with frozen-thawed semen. In the first year (Experiment 1), three sperm numbers per insemination dose (0.25, 0.5 and 1 million sperm) and five sheep farms were used, and in the second year (Experiment 2), parity, age, body weight, body condition score (BCS) and postpartum days were investigated to compare pregnancy and lambing rates. High pregnancy and lambing rates (70.6 and 70.6%, respectively) were obtained with 0.25 million sperm per dose. There were no significant differences in the pregnancy and lambing rates among the five farms, but there was a tendency for one farm to have higher pregnancy (75.8%, P=0.065) and lambing (72.7%, P=0.077) rates than those (46.7–53.3% and 45.2–53.3% for the pregnancy and lambing rates, respectively) of the other farms. In Experiment 2, ewe age significantly affected both the pregnancy and lambing rates. Nulliparous ewes had a higher lambing rate (72.0%) than that (44.2%) of multiparous ewes, but a significant difference was not revealed. Regardless of body weight, BCS tended to be an important factor influencing on fertility of ewes. Body weight and the postpartum days did not affect the fertility of ewes. It was concluded from these results that the fertility of Suffolk ewes after intrauterine insemination with frozen semen was significantly influenced by sperm number per dose and ewe age. Nulliparous ewes at less than three years of age and with a BCS of more than 3.0 are expected to have higher fertility than other ewes.

**Key words:** Artificial insemination, Fertility, Frozen semen, Sheep

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Sheep artificial insemination (AI) with frozen-thawed semen has not fully spread in the field throughout the world, although many studies have been conducted for the AI technologies, such as semen extenders, freezing methods, AI methods and timing. The fertility of ewes inseminated with frozen-thawed semen into the cervical orifice, an ordinal deposition site in sheep AI, is generally low (20-30%). Since the report of Killeen and Caffery [1], only the method of depositing frozen-thawed semen into the uterus with the aid of laparoscopy has resulted in acceptable lambing rates (60-80%). Over the course of a 10-year period (1993 to 2003) in our laboratory, a total of 966 ewes were inseminated with frozen-thawed semen by a fixed-time intrauterine method using laparoscopy during the non-breeding season, and a 54% lambing rate was obtained [2]. However, the fertility (30 to 85% of lambing rate) of ewes after AI varied with physiological status amongst individual ewes, sheep farms, different years and other unknown factors [3–8].

The fertility of ewes after AI is affected by many factors from both the male and female sides. In regard to male factors, timing of AI, insemination doses (numbers of motile spermatozoa) and semen sources from different rams are important, and determination of the optimal sperm number per insemination dose is especially important for optimum fertility under field conditions. On the other hand, female factors include types and duration of progestogen treatment for induction and synchronization of estrus and ovulation, breeding or non-breeding season, body condition score (BCS) and physiological status and age of ewes and different sheep farms. However, it would be always difficult to determine which factor(s) derived from the male and female sides have the most influence on the fertility of ewes inseminated with frozen semen during the non-breeding season. In the field, sheep AI with frozen-thawed semen at a fixed-time basis following treatment with estrus and ovulation induction during the non-breeding season has a major advantage in terms of time and labor when compared with performing estrus detection on non-synchronized ewes.

Therefore, two field trials were conducted during the non-breeding season with the aim of determining the factor(s) affecting on fertility of ewes after intrauterine insemination with frozen-thawed semen using a synthetic semen extender (AndroMed: Minitub, Tiefenbach, Germany). In Experiment 1, three sperm numbers per insemination dose ($100 \times 10^6$, $50 \times 10^6$, $25 \times 10^6$ sperm) and five sheep farms were used, and in Experiment 2, parity (nulliparous or multiparous), age, body weight, BCS and postpartum days were investigated to compare the pregnancy and lambing rates for two successive years.

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## Materials and Methods

The present study was approved by the Animal Experimental Committee of Obihiro University of Agriculture and Veterinary Medicine in accordance with the Guiding Principles for the Care and Use of Research Animals.

*Animals*

Two successive field trials were conducted at Shibetsu Sheep Farm in Hokkaido, Japan, during the non-breeding season (June to July 2008 and July 2009). Overall, 118 and 267 mature Suffolk ewes (1 to 10 years old) respectively, were used for the first (2008: Experiment 1) and second (2009: Experiment 2) trials, respectively. In Experiment 1, the118 ewes belonged to five different sheep farms were brought into a flock at the Shibetsu Sheep Farm a few weeks before the start of the trial. Basically, the ewes during both seasons were fed 3 kg/day of hay (mainly orchards) supplemented with 300 g/day of concentrates (13% crude protein and 76% total digestible nutrients) and were provided with free access to fresh water and mineral blocks throughout the study. For Experiment 2, 267 nulliparous or multiparous ewes were used. The nulliparous ewes were less than 3 years old, and the multiparous ewes had lambing experience in their parities. Also, the ewes were at different postpartum days from the previous lambing. All ewes were kept under the same management manner throughout the experiments.

*Treatment*

Treatments for inducing estrus and ovulation were basically the same in both Experiments 1 and 2. On June 26th, 2008 (Experiment 1), 118 ewes were treated with Controlled Internal Drug Releasing Devices (CIDRs) containing 0.3 g progesterone (Eazi-Breed, type G: Pfizer New Zealand, Auckland, New Zealand). CIDRs were inserted for 12 days into the vagina of the ewes, and 500 IU equine chorionic gonadotropin (eCG, Serotropin; Teikoku-Zoki, Tokyo, Japan) was intramuscularly injected one day before CIDR removal.

The same treatment (CIDR + eCG) was performed for the 267 ewes on three successive days (July 24th, 25th and 26th, 2009) in Experiment 2. Body weight and BCS (1, 2, 3, 4, and 5: very thin, thin, fair, fatty, very fatty, respectively) were measured at the hormonal treatment. The ranges and means (±SEM) of body weight and BCS were 47–114 kg and 75.8 ± 1.0 kg and 1.5–5.0 and 3.2 ± 0.1, respectively.

*Artificial insemination (AI)*

For Experiment 1, semen was collected from a Suffolk ram (3 years old) using an artificial vagina and was diluted 10 to 15 folds in a water bath (30 C) to give an initial sperm concentration of 250 $\times 10^6$/ml. The semen extender used was a synthetic semen extender (AndroMed) imported from Minitub, Tiefenbach, Germany. The dilution and freezing methods were the same as reported in previous studies [8, 9]. In brief, the diluted semen provided $100 \times 10^6$ spermatozoa per 0.4-ml insemination dose. The diluted semen was further extended to sperm concentrations of $125 \times 10^6$/ml and $62.5 \times 10^6$/ml for an insemination dose per head (0.4-ml) containing 50 $\times 10^6$ and $25 \times 10^6$ sperm, respectively.

The diluted semen with three different sperm concentrations ($100 \times 10^6$, $50 \times 10^6$, $25 \times 10^6$) was gradually cooled to 4 C for 2–3 h. The cooled semen was frozen in 0.5-ml straws according to the methods described previously [7–9]. In brief, the semen samples were packed in straws and kept at 4 C before freezing. They were exposed to liquid nitrogen ($LN_2$) vapor (–125 C to –130 C) for 3–4 min, plunged into $LN_2$ (–196 C) and stored in $LN_2$ until use for AI. The frozen straws were thawed at 37 C in a water bath for 20–30 sec, and the motility of the spermatozoa in each straw was evaluated. The straws with a percentage of motile spermatozoa of approximately 50% were used for AI.

In Experiment 1, 11 ewes lost their CIDRs during the insertion period, and they were excluded from the experiment. A fixed-time intrauterine insemination was performed for the remaining 107 ewes. However, in seven of the 107 ewes, intrauterine insemination was not possible due to fatness of the ewes and technical failures. Therefore, the final numbers of ewes inseminated using one of the three sperm numbers per dose ($100 \times 10^6$, $50 \times 10^6$, $25 \times 10^6$ sperm) were 33, 33 and 34 ewes, respectively. An intrauterine AI was carried out 40–49 h after CIDR removal, regardless of the incidence of estrus, by two inseminators. In regard to the insemination dosage (0.4-ml per ewe), the number of motile spermatozoa per ewe was approximately half of the number of spermatozoa contained in the dose ($50 \times 10^6$, $25 \times 10^6$, $12.5 \times 10^6$ per ewe). Half the volume of each insemination dose (0.2-ml) was deposited into each uterine horn using an insemination pipette (No. 20887; I.M.V., Rue Clémenceau, France) with the aid of laparoscopy [3–8].

For Experiment 2, semen collected from four Suffolk rams (3 to 5 years old) and frozen with AndroMed at a sperm concentration of $50 \times 10^6$ ($25 \times 10^6$ motile sperm) per insemination dose (0.4-ml) was used for a fixed-time intrauterine insemination in different types of ewes (nulliparous and multiparous ewes). During CIDR insertion, 29 of the 267 ewes lost their CIDRs and were not inseminated in this experiment. Also, four ewes that retained their CIDRs were not inseminated due to heavy fat tissues in the abdominal cavity. The total number of ewes inseminated was 234 heads, but three of the inseminated ewes died during the 60-day period before pregnancy diagnosis. Therefore, the final number of ewes available for the fertility trials was 231 heads, including 75 nulliparous and 156 multiparous ewes. In this experiment, parity (nulliparous or multiparous), age (3.1 ± 0.1 years old), body weight (75.8 ± 1.0 kg), BCS (3.2 ± 0.1) and postpartum days (152.5 ± 3.5 days) were the main factors to investigate for comparison of fertility after AI. Furthermore, ewe ages were classified into four groups (<3, 3–4, 5–6 and ≥7 years old) of inseminated ewes (143, 46, 26 and 16 heads, respectively). Besides the main factors, there were three other factors that were examined, three successive AI days, three inseminators and the four rams used.

In both Experiments 1 and 2, pregnancy was diagnosed 60 days after AI by real-time ultrasonic scan. Lambing rate (number of ewes lambed/number of ewes inseminated) and prolificacy (number of lambs born/number of ewes lambed) were recorded.

*Statistical analysis*

In both Experiments 1 and 2, the pregnancy and lambing rates

**Table 1.** Effect of sperm number per dose on the fertility of ewes after intrauterine insemination with frozen-thawed semen during the non-breeding season (Experiment 1)

| Sperm numbers ($\times 10^6$) | No. ewes | | | Prolificacy* |
|---|---|---|---|---|
| | Inseminated | Pregnant (%) | Lambed (%) | |
| 100 | 33 | 21 (63.6)[a,b] | 20 (60.6)[a,b] | 1.75 ± 0.12 |
| 50 | 33 | 13 (39.4)[a] | 12 (36.4)[a] | 1.83 ± 0.16 |
| 25 | 34 | 24 (70.6)[b] | 24 (70.6)[b] | 1.88 ± 0.16 |

* Numbers of lambs born relative to the numbers of ewes lambed (Mean ± SEM). [a,b] Values with different superscripts are significantly different (P<0.05).

**Table 2.** Effect of sheep farm on the fertility of ewes after intrauterine insemination with frozen-thawed semen during the non-breeding season (Experiment 1)

| Farms | No. ewes | | | Prolificacy* |
|---|---|---|---|---|
| | Inseminated | Pregnant (%) | Lambed (%) | |
| A | 15 | 7 (46.7) | 7 (46.7) | 1.29 ± 0.17 |
| B | 15 | 8 (53.3) | 8 (53.3) | 1.88 ± 0.21 |
| C | 6 | 3 (50.0) | 3 (50.0) | 1.33 ± 0.27 |
| D | 31 | 15 (48.4) | 14 (45.2) | 2.00 ± 0.23 |
| E | 33 | 25 (75.8) | 24 (72.7) | 1.92 ± 0.10 |

* Numbers of lambs born relative to the numbers of ewes lambed (Mean ± SEM).

were expressed by the percentages of pregnant and lambed ewes to the inseminated ewes. Data were analyzed using the Statistical Analysis System (SAS; SAS Institute, Cary, NC, USA) software. The data for the pregnancy and lambing rates were analyzed by a factorial design using a logistic regression following a binomial distribution. The main factors in Experiments 1 and 2 were sperm numbers per dose and sheep farms, and parity (nulliparous or multiparous), ewe age, body weight, BCS and postpartum days, respectively. The data were analyzed using the following model: ln $(\alpha/1-\alpha)=\beta+$ main factors + their interactions, where $\alpha=$ frequency of positive outcome and $\beta=$ the intercept. As there were no significant interactions between and among the main factors in both Experiments 1 and 2, they were excluded from the final model. If the main factor had a significant effect, comparisons among the sub-groups were performed using the 95% confidence interval of the odds ratio.

In both Experiments 1 and 2, comparisons of prolificacy (number of lambs born/number of ewes lambed) were conducted by the GLM procedure. Differences were considered significant when the P value was less than 0.05.

## Results

*Experiment 1*

The pregnancy and lambing rates in the ewes inseminated with the three different numbers of spermatozoa are shown in Table 1. There were significant differences in the pregnancy (P=0.008) and lambing (P=0.005) rates among the three sperm concentrations. In the ewes inseminated with $25 \times 10^6$, fertility (70.6 and 70.6% for the pregnancy and lambing rates, respectively) was significantly (P<0.05) higher than for ewes (39.4 and 36.4% for pregnant and lambing rates, respectively) inseminated with $50 \times 10^6$. No significant differences in the pregnancy and lambing rates were found between the $100 \times 10^6$ and $25 \times 10^6$ and between $100 \times 10^6$ and $50 \times 10^6$ sperm concentrations.

The pregnancy and lambing rates at the five different farms (A to E) are shown in Table 2. There were no significant differences in the pregnancy and lambing rates among the farms, but there was a tendency for one farm (E) to have higher pregnancy (75.8%, P=0.065) and lambing (72.7%, P=0.077) rates than those (46.7–53.3 and 45.2–53.3% for the pregnancy and lambing rates, respectively) of the other farms. At farm "E", 28 of the 33 inseminated ewes were less than 2 years old, and 25 (89.3%) and 22 (78.6%) heads of the inseminated ewes were pregnant and lambed, respectively.

Prolificacy was not significantly different among the three sperm numbers/dose (P=0.511) and five farms (P=0.100).

*Experiment 2*

Before analyzing the main factors in this experiment, we confirmed that the three successive days (84, 63 and 84 ewes/day), three inseminators (114, 106 and 11 ewes/inseminator) and four rams (85, 85, 51 and 10 ewes/ram) did not significantly affect the pregnancy and lambing rates, which were 59.5 and 57.1%, 52.4 and 50.8% and 51.2 and 51.2% for the three successive days (P= 0.308 and P=0.448, respectively); 57.9 and 57.0%, 50.9 and 49.1% and 54.5 and 54.5% for the three inseminators (P=0.583 and P=0.509, respectively); and 56.5 and 55.3%, 52.9 and 51.8%, 51.0 and 49.0% and 70.0 and 70.0% for the four rams (P=0.523 and P=0.524, respectively).

Table 3 shows the statistical results for the factors influencing on fertility in the experiment. Ewe age significantly (P<0.001) affected both the pregnancy and lambing rates. The estimated

**Table 3.** Statistical results for factors influencing fertility and prolificacy in Experiment 2

| Factors# | Mean ± SEM | Range | Pregnant* | Lambing* | Prolificacy* |
|---|---|---|---|---|---|
| Parity | — | — | 0.3639 | 0.5216 | 0.3245 |
| Age (years) | 3.1 ± 0.1 | 1.2–10.4 | 0.0007 | 0.0010 | 0.9314 |
| Body weight (kg) | 75.8 ± 1.0 | 47–114 | 0.9484 | 0.8425 | 0.0815 |
| BCS | 3.2 ± 0.1 | 1.5–5.0 | 0.0728 | 0.0673 | 0.1806 |
| Postpartum days | 152.5 ± 3.5 | 62–339 | 0.6265 | 0.8120 | 0.7049 |

# Each factor was calculated using a total of 231 ewes including 75 and 156 nulliparous and multiparous ewes, respectively. *P values.

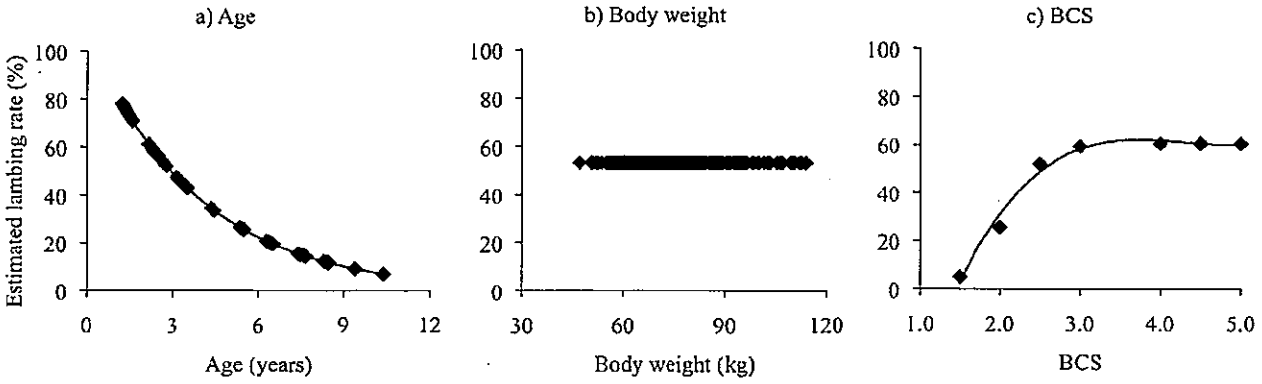


**Fig. 1.** Estimated lambing rates of the inseminated ewes (n=231) in Experiment 2 according to age (a), body weight (b) and BCS (c).

**Table 4.** Effect of ewe age on the fertility of ewes after intrauterine insemination with frozen-thawed semen during the non-breeding season (Experiment 2)

| Ewe's age (years old) | BCS (Mean ± SEM) | No. ewes | | | Prolificacy* |
|---|---|---|---|---|---|
| | | Inseminated | Pregnant (%) | Lambed (%) | |
| <3 | 3.3 ± 0.1 | 143 | 100 (69.9) [a] | 98 (68.5)[a] | 1.48 ± 0.24 |
| 3–4 | 2.9 ± 0.1 | 46 | 13 (28.3) [b] | 13 (28.3)[b] | 1.62 ± 0.13 |
| 5–6 | 3.3 ± 0.2 | 26 | 10 (38.5) [b] | 9 (34.6)[b] | 1.56 ± 0.17 |
| >7 | 3.2 ± 0.2 | 16 | 3 (18.8) [b] | 3 (18.8)[b] | 1.33 ± 0.27 |

A total of 231 ewes (75 nulliparous ewes <3 years old and 156 multiparous ewes including 68 ewes <3 years old) were inseminated. * Numbers of lambs born relative to the numbers of ewes lambed (Mean ± SEM). [a, b] Values with different superscripts are significantly different (P<0.05).

lambing rates showed that the high lambing rate (approximately 50%) in the ewes less than 3 years old gradually declined with increased age (Fig. 1a). Table 4 shows in detail that the ewes less than 3 years old had significantly (P<0.05) higher pregnancy (69.9%) and lambing (68.5%) rates than those (18.8–38.5% and 18.8–34.6% for the pregnancy and lambing rates, respectively) of the ewes at other ages. Furthermore, prolificacy was not significantly different among the ewes in the different age groups (1.33 ± 0.27 to 1.62 ± 0.13).

Nulliparous ewes had a higher lambing rate (72.0%: 54/75 heads) than that (44.2%: 69/156 heads) of multiparous ewes, but a significant difference was not revealed. Prolificacy was also not significantly different between the nulliparous and multiparous ewes (1.50 ± 0.08 and 1.49 ± 0.07, respectively).

Body weight did not affect the pregnancy and lambing rates of the inseminated ewes (Table 3 and Fig. 1b), although there was a tendency (P=0.0815) showing that prolificacy may be influenced by body weight. On the other hand, regardless of body weight, BCS tended to be an important factor influencing on the fertility of ewes after AI (P=0.07 for both the pregnancy and lambing rates) as shown in Table 3. Fig. 1c showed that the estimated lambing rate of the inseminated ewes increased along with increasing BCS up to 3.0 and that the ewes with a BCS of more than 3.0 tended to maintain a high level of fertility (about 60%). Postpartum days did not affect the fertility and prolificacy of the inseminated ewes.

## Discussion

It has been considered that the fertility of ewes after AI is affected by many factors, such as breeding or non-breeding season, methods of hormonal treatment and AI timing. In the present two successive AI trials, female factors such as parity (nulliparous or multiparous), sheep farm, ewe age, body weight, BCS and postpartum days, along with sperm numbers per AI dose as a representative male factor, were investigated in relation to fertility after laparoscopic AI with frozen-thawed semen. Currently, laparoscopic AI on a fixed-time basis is performed at synchronized estrus during the breeding season or at induced estrus during the non-breeding season [5–8,10]. Among the factors tested in the present study, there are various factors that may be correlated with each other, such as age and body weight and body weight and BCS. For examples, young ewes are lighter than aged ewes, and heavy ewes are likely to have a high BCS, although there are some exceptions. However, as no interactions between and among the main factors were revealed in the present study, the interaction term was excluded from the present analyses. In Experiment 2, before analyzing the main factors, we confirmed that there were no significant differences in the other three items likely influencing the fertility of the ewes. These were the AI days (3 days), artificial inseminators (3 persons), and the four different rams.

Among the male factors affecting the fertility of ewes, individual ram difference [11,12], quality (motility, viability and others) and quantity (volume and number of spermatozoa per insemination dose, etc.) of semen for AI are the most important to be considered. In Experiment 1, only one ram was used to avoid any influences on fertility in the 100 ewes by different rams. However, in Experiment 2, in which we used a large number of ewes, frozen-thawed semen derived from four rams were inseminated by three inseminators, and fortunately, there was no significant difference in the fertility of the ewes among the inseminators and the rams used. It has been reported that there is no significant difference on the fertility of ewes inseminated intracervically with fresh or frozen-thawed semen in some field trials [13, 14]. In contrast, there are some reports showing differences in fertility of ewes following cervical AI with semen from different rams [15–21] and ejaculates within a ram [18, 22]. Salamon and Maxwell [10] described that ram differences could be both genetic and environmental, whereas ejaculates differences are probably due to nutrition, management and previous frequency of ejaculation. The fact that there was no significant difference in the fertility among the four rams in the present study may have derived from the fact that the present insemination method was intrauterine AI instead of cervical AI, which may cause clear differences in sperm transport, motility and viability leading to fertilization in individual ram spermatozoa.

Sperm number per ewe inseminated is dependent on the semen deposition site and type of semen (fresh, cooled, frozen). For inseminating into the cervical orifice, 100 to 200 $\times 10^6$ spermatozoa are required for acceptable fertility following cervical or vaginal AI [19, 20, 23–25]. On the other hand, intrauterine AI allows for a decrease in the number of spermatozoa deposited compared with cervical AI [10]. In fact, for intrauterine AI in sheep, an insemination dose containing 50 to 100 $\times 10^6$ spermatozoa has been widely used, including by our laboratory [26, 27]. However, the result in Experiment 1 showing that the low sperm number per insemination dose (25 $\times 10^6$) produced the highest fertility compared with those obtained with the higher sperm numbers per dose (100 $\times$ and 50 $\times 10^6$) was unexpected. However, this result coincides with the reports by Maxwell [16, 17, 28] showing that the minimum sperm number for an intrauterine AI is 20 $\times 10^6$ to yield lambing rates of 39 to 77% in Australian Merino ewes. de Graaf *et al.* [29] also obtained similar lambing rates (48.6, and 36.1%, respectively) by an intrauterine AI with 15 $\times 10^6$ non-sorted frozen-thawed and sex-sorted and re-frozen-thawed ram spermatozoa. Recently, Beilby *et al.* [30] performed laparoscopic insemination with sex-sorted and non-sorted doses of 1 or 15 $\times 10^6$ motile, frozen-thawed ram semen and reported that the pregnancy rates were similar (49%) for both the sorted and non-sorted semen with 15 $\times 10^6$ motile spermatozoa, but the sorted semen with 1 $\times 10^6$ had a significantly higher rate of pregnancy than the non-sorted semen (37 and 16%, respectively). They reinforced the importance of insemination time when the sperm dose is low. The reason for the low fertility using 50 $\times 10^6$ sperm per insemination dose in Experiment 1 was not clear, but the above [16, 17, 28] and present results indicate that a higher number of spermatozoa per insemination dose may be unnecessary if AI is performed timely. The decrease in the sperm number per insemination would lead to increase of the number of ewes inseminated, which is very helpful to sheep AI programs in the field. The insemination dose of 20 or 25 $\times 10^6$ sperm is the almost same as that in routine bovine AI, in which semen is deposited into the uterine body. In the present trials, ewes were treated during the non-breeding season to induce estrus and ovulation for conception, and AI was performed only once on a fixed-time base without estrus detection. The subsequent fertility (pregnancy and lambing rates) after intrauterine insemination of the ewes was likely similar or higher than the recent fertility (40–60%) in cows or heifers.

In Experiment 1, there was a tendency showing that farm difference might be a factor influencing the fertility of inseminated ewes. We understood that individual sheep farms basically fed to ewes with the same feeds, but there was somewhat of a difference in the volume and management policies. Paulenz *et al.* [19, 20] reported that the fertility of ewes inseminated by cervical AI with liquid semen was significantly different among farms, but that the ewe age had no significant effect. In our previous AI study [31] using cervical AI with fresh undiluted semen during the non-breeding season, we also found a farm-related difference in the lambing rates of three farms. In one of the three farms, the mean age of the ewes was higher (5.8 $\pm$ 1.7 years old, n=88) and the BCS was lower (2.9 $\pm$ 0.5) than the ewes in the other two farms (4.4 $\pm$ 1.5 and 3.8 $\pm$ 1.4 years old for age, and 3.4 $\pm$ 0.6 and 3.3 $\pm$ 0.5 for BCS). The lambing rate (33.7%) of the ewes at that farm was significantly lower than those of the other two farms (50.0 and 61.7%, respectively). Actually, in Experiment 1, the high fertility at Farm E resulted from the fact that a large number (28 out of 33) of the inseminated ewes was less than 2 years old, and 25 (89.3%) and 22 (78.6%) of those ewes was pregnant and lambed. However, it has been reported that in comparison with adult and aged ewes, the fertility of young and maiden ewes have lower fertility due to impaired sperm transport combined with low mucus production in the cervical canals during

estrus [32, 33]. Also, among various factors influencing fertility after AI in cattle, age, BCS and physiological status of the individual animal such as heifers and high-milking dairy cows, are most important [34]. Also, in goat AI, nulliparous goats are less fertile than multiparous goats when intracervical AI is performed. The lower fertility rates observed in nulliparous goats were explained by the time of cervical AI relative to ovulation [35, 36]. However, this explanation can be excluded when an intrauterine AI is performed in sheep or goats. In a recent study using *Bos indicus* cows, Sa Filho *et al.* [37] investigated several factors affecting fertility after a fixed-time AI, similar to our present study, and reported that farms, breeds, BCS, sires and AI technicians significantly affected the pregnancy rates, but that postpartum days did not. Paulenz *et al.* [20] reported that age of the ewes had a significant effect on the non-return rate, but not on the lambing rate. In our present trials, both the pregnancy and lambing rates in the ewes significantly declined with increased aged. This could be understandable based on the facts that aged ewes have increased risks of reproductive disorders and decreased rates of ovulation with quality ovulated oocytes compared with young ewes. For BCS, ewes with a BCS of 2.5 to 3.0 are generally used for natural or artificial breeding [38, 39]. Even in tropical West African ewes with a body weight of 36.7 ± 0.4 kg, the mean of BCS before AI is 2.9 ± 0.1 [39]. In Experiment 2 (Table 4), the mean BCS was similar (2.9 to 3.3) among the different ages of the 231 inseminated ewes, but BCS showed a tendency to affect fertility. Taken together, the above results suggest that body nutritional condition would be an important factor, next to ewe age, influencing the fertility of ewes after AI, regardless of body weight.

In the present trials, body weight did not significantly affect fertility, which was an unexpected result. Because it has been traditionally accepted that, compared with lighter ewes, ewes with a high body weight have higher ovulation rates, leading to higher fertility. In this experiment, there was only a tendency showing that body weight may influence on prolificacy. Postpartum days also did not affect the fertility and prolificacy of inseminated ewes in the present study. However, in our unpublished data, the lambing rate of 65 ewes inseminated over 60 days after weaning was significantly higher than that of 72 ewes inseminated within 60 days after weaning (60.0 and 34.7%, respectively). As weaning in sheep is usually terminated at 3 to 4 months after lambing, it would be desirable to perform AI for out-of-seasonal breeding at 5 to 6 months after lambing.

In general, Suffolk rams are less fertile than Dorset rams in out-of-seasonal breeding. Ewe breed types are also a significant source of variation in fertility of ewes inseminated artificially [10, 13, 21, 40]. Differences in the mean time of ovulation and ovulation rates in different breeds of ewes at different locations may explain the variations in fertility [10]. Suffolk ewes are less fertile compared with other breeds of ewes, such as Dorset and Finnish Landrace ewes. Prolific sheep, such as the Booroola Merino, Finnish Landrace and Romanov, are more fertile due to the higher ovulation rates than non-prolific Suffolk or Ile-de-France ewes [13]. Although only Suffolk ewes were used in the present field studies, our unpublished data on fertility of 58 ewes intracervically inseminated with fresh-diluted semen showed that the lambing rate was only 33% with two inseminations per estrus. Therefore, it is feared that the fertility of Suffolk ewes achieved by cervical AI with frozen-thawed semen would be lowered much more and that cervical AI could not be applied in the fields. At present, laparoscopic AI is the only method that ensures satisfactory fertility using frozen-thawed semen in sheep including Suffolk ewes under field conditions. To increase the fertility of Suffolk ewes by laparoscopic AI with frozen-thawed semen, it is important to select healthy ewes individually based on their physiological or nutritional conditions, especially age and BCS in relation to body weight and parity.

In the present study, we used frozen-thawed ram semen diluted with a synthetic semen extender, "Andromed" that is free of animal-derived ingredients and the use of this semen extender is becoming a widespread recommendation. The present fertility trials by intrauterine AI in sheep have confirmed that the fertility results using "AndroMed" are comparable to those of semen extenders containing egg yolk or bovine serum albumin [7–9].

In conclusion, the present field studies showed that the fertility of Suffolk ewes after intrauterine insemination with frozen semen by laparoscopy was significantly influenced by sperm number per AI dose and ewe age. Nulliparous ewes less than 3 years old and with a BCS of more than 3.0 are expected to have higher fertility than other types of ewes.

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## References

1. Killeen ID, Caffery GJ. Uterine insemination of ewes with the aid of laparoscope. *Aust Vet J* 1982; 59: 95.
2. Fukui Y. New Reproduction Technologies in Sheep. Y Fukui (ed.), Tokyo: Tokyo Agricultural University Press; 2004: 75–80 (in Japanese).
3. Fukui Y, Hirai H, Honda K, Hayashi K. Lambing rates by fixed-time intrauterine insemination with frozen semen in seasonally anestrous ewes treated with two different progestogen-impregnated sponge or CIDR devices. *J Reprod Dev* 1993; 39: 1–5.
4. Fukui Y, Fujii M, Tashiro Y. Insemination doses of frozen-thawed semen in seasonally anestrous ewes treated with two different progesterone-impregnated intravaginal devices. *J Reprod Dev* 1993; 39: 269–273.
5. Fukui Y, Tabuchi K, Yamada A, Hayashi N, Tanaka K. Effect of insertion periods of controlled internal drug release device (CIDR) on conception rate by fixed-time intrauterine insemination with frozen semen in seasonally anestrous ewes. *J Reprod Dev* 1994; 40: 221–226.
6. Fukui Y, Iida K, Okada A, Zyouzyou Y, Wach S, Togawa M. Fertility of estrus-induced ewes during the non-breeding season and artificially inseminated with frozen semen imported from New Zealand. *J Reprod Dev* 2002; 48: 485–488.
7. Fukui Y, Kohno H, Togari T, Hiwasa M. Fertility of ewes inseminated intrauterinally with frozen semen using extender containing bovine serum albumin. *J Reprod Dev* 2007; 53: 959–962.
8. Fukui Y, Kohno H, Togari T, Hiwasa M, Okabe K. Fertility after artificial insemination using a soybean-based semen extender in sheep. *J Reprod Dev* 2008; 54: 286–289.
9. Hiwasa M, Kohno H, Togari T, Okabe K, Fukui Y. Fertility after different artificial insemination methods using a synthetic semen extender in sheep. *J Reprod Dev* 2009; 55: 50–54.
10. Salamon S, Maxwell WMC. Frozen storage of ram semen II. Causes of low fertility after crervical insemination and methods of improvement. *Anim Reprod Sci* 1995; 38: 1–36.
11. Fukui Y, Kobayashi M, Ono H. Effects of injection time of pregnant mare's serum gonadotropin and individual rams on fertility of ewes in a trial of out-of-season breeding. *Jpn J Anim Reprod* 1985; 31: 16–24.

12. **Fukui Y, Tsubaki M, Kobayashi M, Ono H.** Mating behavior of rams on ewes at induced estrus during the non-breeding season. *Jpn J Anim Reprod* 1986; 32: 195–201.
13. **Donovan A, Hanrahan JP, Kummen E, Duffy P, Boland MP.** Fertility in the ewe following cervical insemination with fresh or frozen-thawed semen at a natural or synchronized oestrus. *Anim Reprod Sci* 2004; 84: 359–368.
14. **Ward F, Wade M, Evans ACO, Longergan P.** Relationship between *in vitro* sperm functional tests and *in vivo* fertility of rams following cervical artificial insemination of ewes with frozen-thawed semen. *Theriogenology* 2008; 69: 513–522.
15. **Colas G.** Fertility in the ewe after artificial insemination with fresh and frozen semen at the induced oestrus, and influence of the photoperiod on the semen quality of the ram. *Live Prod Sci* 1979; 6: 153–166.
16. **Maxwell WMC.** Artificial insemination of ewes with frozen-thawed semen at a synchronized oestrus. I. Effect of time of onset of oestrus, ovulation, and insemination on fertility. *Anim Reprod Sci* 1986; 10: 301–308.
17. **Maxwell WMC.** Artificial insemination of ewes with frozen-thawed semen at a synchronized oestrus. I. Effect of dose of spermatozoa and site of insemination on fertility. *Anim Reprod Sci* 1986; 10: 309–316.
18. **Windsor DP.** Variation between ejaculates in the fertility of frozen ram semen used for cervical insemination of merino ewes. *Anim Reprod Sci* 1997; 47: 21–29.
19. **Paulenz H, Adnoy T, Fossen OH, Soderquist L, Berg KA.** Effect of deposition site and sperm number on the fertility of sheep inseminated with liquid semen. *Vet Rec* 2002; 150: 299–302.
20. **Paulenz H, Adnoy T, Soderquist L.** Comparison of fertility results after vaginal insemination using different thawing procedures and packages for frozen ram semen. *Acta Vet Scan* 2007; 49: 26–32.
21. **Papadopoulos S, Hanrahan JP, Donovan AS, Duffy P, Boland MP, Lonergan P.** *In vitro* fertilization as a predictor of fertility from cervical insemination in sheep. *Theriogenology* 2005; 63: 150–159.
22. **Smith JF, Murray GR.** Use of bovine oocytes for evaluation of ram semen. *Proc NZ Anim Prod* 1996; 56: 304–306.
23. **Paulenz H, Soderquist L, Adnoy T, Fossen OH, Berg KA.** Effect of milk- and TRIS-based extenders on the fertility of sheep inseminated vaginally once or twice with liquid semen. *Theriogenology* 2003; 60: 759–766.
24. **Paulenz H, Soderquist L, Adnoy T, Nordstoga A, Gulbrandsen B, Berg KA.** Fertility results after different thawing procedures for ram semen frozen in minitubes and mini straws. *Theriogenology* 2004; 61: 1719–1727.
25. **Paulenz H, Soderquist L, Adnoy T, Nordstoga A, Berg KA.** Effect of vaginal and cervical deposition of semen on the fertility of sheep inseminated with frozen-thawed semen. *Vet Rec* 2005; 156: 372–375.
26. **Fukui Y, Ishikawa D, Ishida N, Okada M, Itagaki R, Ogiso T.** Comparison of fertility of estrous synchronized ewes with four different intravaginal devices during the breeding season. *J Reprod Dev* 1999; 45: 337–343.
27. **Fukui Y, Itagaki R, Ishida N, Okada M.** Effect of different hCG treatment on fertility of estrus-induced and artificially inseminated ewes during the non-breeding season. *J Reprod Dev* 2001; 47: 189–195.
28. **Maxwell WMC.** Current problems and future potential of artificial insemination programmes. In: Lindsay DR, Pearce DT (eds.), Reproduction in Sheep. Cambridge : Cambridge University Press, 1984; 291–297.
29. **de Graaf SP, Evans G, Maxwell WMC, Cran DG, O'Brien JK.** Birth of offspring of pre-determined sex after artificial insemination of frozen-thawed, sex-sorted and re-frozen-thawed ram spermatozoa. *Theriogenology* 2007; 67: 391–398.
30. **Beilby KH, Grupen CG, Thompon PC, Maxwell WMC, Evans G.** The effect of insemination time and sperm dose on pregnancy rate using sex-sorted ram semen. *Theriogenology* 2009; 71: 835–839.
31. **Fukui Y, Yamamoto Y, Goda S, Ono H.** Single or double inseminations at fixed-time basis on lambing rate of ewes treated with progestogen-impregnated intravaginal sponges during the non-breeding season. *Jpn J Anim Reprod* 1991; 37: 231–235.
32. **Selaive-Villarroel AB, Kennedy JP.** Fertility in young and mature merino ewes: 1. Cervical mucus production. *Theriogenology* 1983; 20: 537–541.
33. **Selaive-Villarroel AB, Kennedy JP.** Fertility in young and mature merino ewes: 2. Sperm transport. *Theriogenology* 1983; 20: 543–547.
34. **Schenk JL, Cran DG, Everett RW, Seidel Jr. GE.** Pregnancy rates in heifers and coed with cryopreserved sexed sperm: Effect of sperm numbers per inseminate, sorting pressure and sperm storage before sorting. *Theriogenology* 2009; 71: 717–728.
35. **Leboeuf B, Restall B, Salamon S.** Production and storage of goat semen for artificial insemination. *Anim Repod Sci* 2000; 62: 113–141.
36. **Batista M, Nino T, Alamo D, Castro N, Santana M, Gonzalez F, Cabrera F, Gracia A.** Successful artificial insemination using semen frozen and stored by an ultrafreezer in the Majorera goat breed. *Theriogenology* 2009; 712: 1307–1315.
37. **Sa Filho OG, Meneghetti M, Peres RFG, Lamb GC, Vasconcelos JLM.** Fixed-time artificial insemination with estradiol and progesterone for *Bos indicus* cows II. Strategies and factors affecting fertility. *Theriogenology* 2009; 72: 210–218.
38. **Husein MQ, Ababneh MM.** A new strategy for superior reproductive performance of ewes bred out-of-season utilizing progestagen supplement prior to withdrawal of intravaginal pessaries. *Theriogenology* 2008; 69: 376–383.
39. **Contreras-Solis I, Vasquaz B, Diaz Y, Letelier C, Lopez-Sebastian A, Gonzalez-Bulnes A.** Efficiency of estrous synchronization in tropical sheep by combining short-interval cloprostenol-based protocols and "male effect". *Theriogenology* 2009; 71: 1018–1025.
40. **Fukui Y, Kohno H, Togari T, Matsuoka D, Imai H.** Effects of insemination time, breed, and inseminator on fertility of ewes intrauterinally inseminated with frozen-thawed semen imported from New Zealand. *Reprod Fertil Dev* 2007; 19: 123 (no. 10, Abstract).