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—Technical Note—

Fertility after Artificial Insemination Using a Soybean-Based Semen Extender in Sheep

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Abstract. The present study aimed to compare the fertility of ewes intrauterinally inseminated with frozen-thawed semen using a soybean-based semen extender (AndroMed) with those of ewes intrauterinally inseminated with frozen-thawed semen using a Tris-based extender containing either egg yolk or BSA. Suffolk ewes (n=104) were treated with an intravaginal sponge containing 40 mg fluoroprogesterone acetate (FGA) for 12 days and an intramuscular injection of 500 IU equine chorionic gonadotropin to induce estrus and ovulation during the non-breeding season (July, 2007). Intrauterine insemination was carried out 40–46 h after removal of the FGA sponge (n=90), regardless of the incidence of estrus. The pregnancy rates were not significantly different among the semen extenders containing egg yolk (64.5%) or BSA (58.6%) and AndroMed extender (56.7%). The lambing rates (64.5, 55.2 and 56.7% for the semen extenders containing egg yolk, BSA and AndroMed, respectively) and prolificacy (1.59 to 1.75) were also not significantly different. The present results indicate that an egg yolk-containing semen extender can be replaced with the non-animal derived extender AndroMed, which could be used for intrauterine insemination using frozen-thawed ram semen without reducing fertility.

Key words: Artificial insemination, Fertility, Semen extender, Sheep

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Egg yolk-based semen extenders have been widely utilized for cryopreservation of semen from farm animals including sheep [1–3]. However, it is not always easy to prepare semen extenders consistent with quality standards because of the individual quality differences inherent in egg yolk due to the numbers of days after laying and the storage period. Also, addition of egg yolk reduces the acrosome integrity of goat spermatozoa [4], and high egg yolk concentrations reduce the post-thawing viability of ejaculated spermatozoa in several species, such as goats [5], rams [1] and water buffaloes [6]. Furthermore, there has been movement recently to eliminate all animal ingredients, including egg yolk, milk or bovine serum albumin (BSA), in order to design a defined semen extender. Removal of chicken egg yolk from semen extenders would provide several advantages, such as improved consistency in the components of semen extenders and elimination of hygienic risks. Therefore, development of a synthetic semen extender free of animal sources has been desired. A soybean lecithin-based extender (AndroMed; Minitub, Tiefenbach, Germany) has been developed and utilized for bovine [7–9] and mountain gazelle semen [10]. Fresh and frozen ram semen diluted with a synthetic semen extender, AndroMed, has been inseminated with satisfactory fertility results in Norway (Paulenz H.: personal communication).

Low fertility (20–30%) in ewes inseminated with frozen semen into the cervical orifice, an ordinal deposition site for artificial insemination (AI) in sheep, has not been applied fully on the field, except for intrauterine AI using laparoscopy (60–80% in lambing rates) [11–15]. In Norway, Paulenz *et al*. [16, 17] inseminated frozen ram semen into either the cervical orifice or deep vagina and obtained a lambing rate of 70%. However, satisfactory fertility with frozen-thawed ram semen deposited into the cervical orifice or vagina has not been reported in other countries.

The present study aimed to compare the fertility of ewes intrauterinally inseminated with frozen-thawed semen using a synthetic semen extender (AndroMed) with those of ewes intrauterinally inseminated with frozen-thawed semen using Tris-based extender containing egg yolk or BSA.

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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

The present experiment was conducted at Shibetsu Sheep Farm in Hokkaido, Japan, during the non-breeding season (July 2007). A total of 104 mature Suffolk ewes (2–9 years old) were used. Basically, the ewes 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.

Table 1. Fertility after intrauterine insemination with frozen-thawed semen using AndroMed extender

Semen extender containing	No. ewes (%)			No. Lambs: prolificacy (Mean ± SEM)
	Inseminated	Pregnant	Lambed	
Tris/Egg yolk	31	20 (64.5)	20 (64.5)	35 (1.75 ± 0.14)
Tris/BSA	29	17 (58.6)	16 (55.2)	27 (1.69 ± 0.15)
AndroMed	30	17 (56.7)	17 (56.7)	27 (1.59 ± 0.15)
Total	90	54 (60.0)	53 (58.9)	89 (1.68 ± 0.15)

Treatment

On July 13th, one hundred and four ewes were treated with an intravaginal sponge containing 40 mg fluoroprogesterone acetate (FGA; Intervet Internatinal, Boxmeer, Holland). FGA sponges were inserted for 12 days into the vagina of ewes, and 500 IU equine chorionic gonadotropin (eCG, Serotropin; Teikoku Zoki, Tokyo, Japan) was intramuscularly injected one day before removal of the FGA sponges.

Chemical reagents

All chemical reagents for preparation of semen extenders were of the highest purity commercially available. Tris-hydroxymethyl aminomethane (Tris) was purchased from Merck (Darmstadt, Germany). Fructose and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Citric acid and glycerol were purchased from Wako (Osaka, Japan). A synthetic semen extender (AndroMed) was imported from Mini-tub (Tiefenbach, Germany).

Artificial insemination (AI)

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 a final sperm concentration of 250×10^6/ml. Three semen extenders were used: Tris-based extenders containing 297.58 mM Tris, 96.32 mM citric acid, 82.66 mM fructose, 5% (v/v) glycerol and either 15% (v/v) egg yolk [18] or 10% (w/v) BSA [15, 19] and AndroMed. The diluted semen was gradually cooled to 4 C for 2–3 h. The cooled semen was frozen in 0.25-ml straws according to the methods described previously [19]. 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.

Fourteen ewes lost their FGA sponges during the insertion period, and they were excluded from the experiment. A fixed-time intrauterine insemination was performed for the remaining 90 ewes using one of the three types of frozen-thawed semen (31, 29 and 30 ewes for the semen extenders containing egg yolk, BSA and AndroMed, respectively). Intrauterine AI was carried out 40–46 h after removal of the FGA sponge, regardless of the incidence of estrus by two inseminators (A and B). In regard to the insemination dosage (0.4 ml per ewe), the numbers of spermatozoa per ewe was approximately 100×10^6, and the number of motile spermatozoa per ewe was approximately half that (50×10^6 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 [11–15].

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

The pregnancy and lambing rates of the inseminated ewes were analyzed using the chi-square test. Prolificacy was compared using Student's *t*-test. A value of $P<0.05$ was chosen as an indication of significance.

Results

As shown in Table 1, the pregnancy rate was not significantly different among the semen extenders containing egg yolk (64.5%) or BSA (58.6%) and the synthetic extender (AndroMed: 56.7%). The pregnancy (63.4 and 55.6% for inseminators A and B, respectively) and lambing rates (61.7 and 55.6%, respectively) were not significantly different for the two inseminators. The lambing rates (64.5, 55.2 and 56.7% for semen extenders containing egg yolk, BSA and AndroMed, respectively) and prolificacy (1.75, 1.69 and 1.59, respectively) were also not significantly different.

Discussion

The present study was the first attempt of AI with frozen-thawed ram semen using a soybean lecithin-based extender, AndroMed, in Japanese sheep. The commercially available AndroMed (Tiefenbach, Germany) is a Tris-based buffer containing phospholipids, citric acid, sugars, antioxidant and glycerol, and has been utilized for cyropreservation of bovine [7–9] and mountain gazelle semen [10]. Fresh and frozen ram semen diluted with AndroMed have been inseminated with satisfactory fertility results in Norway (Paulenz H.: personal communication). Anel *et al*. [20] reported that the soybean lecithin in AndroMed contains a high content of egg yolk-like phospholipids. Egg yolk is a main component in extenders for storage and freezing of most mammalian semen including that of sheep. The active fraction of egg yolk is believed to be a low-density lipoprotein [1]. However, possible disadvantages of

using egg yolk, such as great variability of composition and the risk of microbiological contamination, have also been pointed out [21]. Furthermore, it has been reported that addition of egg yolk reduces the acrosome integrity and the post-thawing viability of ejaculated spermatozoa in several species, such as goats [4, 5], rams [1] and water buffaloes [6]. Our recent study [15] showed that a semen extender containing10% BSA without egg yolk resulted in acceptable fertility that is similar to that achieved with extender containing egg yolk (63 and 67% lambing rates for BSA- and egg yolk-containing extenders, respectively). Although addition of BSA demonstrated to develop a synthetic, semi-defined semen extender in place of egg yolk, complete elimination of egg yolk or BSA from semen extender and the use of a non-animal derived compound, such as a soybean lecithin instead is a better choice for reducing the hygienic risks of contamination of semen doses with bacteria and mycoplasma (AndroMed [21] or Biociphos plus [22]). The present AI trial confirmed the fertility results using a BSA-containing extender for freezing ram semen and demonstrated that a completely defined, synthetic semen extender (AndroMed) could be used for sheep AI with frozen-thawed semen.

Low fertility (20–30%) in ewes inseminated with frozen semen into the cervical orifice, an ordinal deposition site in sheep AI, has not been applied fully on the field. In 1973, Andersen *et al*. [23] first attempted intrauterine AI in sheep with frozen semen through cervical penetration and obtained conception rates of 89 and 54% in natural and synchronized estrous ewes, respectively. The morphometric parameters, such as the external diameter and number of cervical folds, and types of cervical orifice are strongly dependent on breed and age of the ewes [24–26]. Fukui [25] and Fukui and Roberts [26, 27] attempted the non-surgical intrauterine insemination with frozen semen and obtained satisfactory fertility. However, the intrauterine insemination through cervical penetration is time-consuming, and successful penetration is dependent on breed and age of the ewes which have varying external diameters, number of cervical folds, and types of cervical orifice. In 1982, ovine intrauterine insemination by laparoscopy was developed in Australia [28]. Since then, technologies related to laparoscopic AI have spread throughout the world, and at present, laparoscopic AI is the only method that ensures intrauterine application of frozen-thawed semen with satisfactory fertility in sheep under field conditions [11–15]. However, laparoscopic AI has problems related to its complexity, high equipment costs and the need for trained technicians and other problems related to animal welfare [20]. The best insemination method is a simple, practical method, such as vaginal insemination (shot in dark). Olesen [29] and Paulenz *et al*. [16, 17] inseminated frozen ram semen into either the cervical orifice or deep vagina and obtained high fertility results (58 and 72% lambing rates). However, satisfactory fertility with frozen-thawed ram semen deposited into the cervical orifice or vagina has not been reported in other countries. Therefore, it is worthwhile to attempt cervical or vaginal AI with ram semen frozen using AndroMed at various sheep farms in future trials.

In conclusion, the present field trials have demonstrated that an egg yolk-containing semen extender can be replaced with the synthetic extender, AndroMed, which could be used for intrauterine insemination using frozen-thawed ram semen without reducing fertility.

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References

1. **Watson PF, Martin IC**. The influence of some fractions of egg yolk on the survival of ram spermatozoa at 5 degrees C. *Aust J Biol Sci* 1975; 238: 145–152.
2. **Watson PF**. The role of lipid and protein in the protection of ram spermatozoa at 5 C by egg yolk lipoprotein. *J Reprod Fertil* 1981; 2: 337–340.
3. **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: 291–298.
4. **Aboagla EM, Terada T**. Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. *Theriogenology* 2004; 62: 1160–1172.
5. **Ritar AJ, Salamon S**. Effects of month of collection, method of processing, concentration of egg yolk and duration of frozen storage on viability of Angoras goat spermatozoa. *Small Rumin Res* 1991; 4: 29–37.
6. **Kumar S, Sahni KL, Mohan G**. Effect of different extender formulations on acrosomal maintenance of buffalo spermatozoa frozen in milk, Tris, and sodium-citrate dilutors. *Ind J Anim Sci* 1993; 63: 1233–1239.
7. **Muller-Schlosser F, Hinsch E, Bohm J, Schill WB, Hinsch KD**. Investigations on egg-yolk free diluenting medium for the cryopreservation of bull spermatozoa. *Tierartl Prax* 1995; 23: 363–366.
8. **Van Wagtendonk-de Leeuw AM, Haring RM, Kaal-Lansbergen LMTE, den Dass JHG**. Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. *Theriogenology* 2000; 54: 57–67.
9. **Aires VA, Hinsch KD, Mueller-Schloesser F, Bogner K, Mueller-Schoedder S, Hinsch E**. *In vitro* and *in vivo* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. *Theriogenology* 2003; 60: 269–279.
10. **Saragusty J, Gacitua H, King R, Arav A**. Post-mortem semen cryopreservation and characterization in two different endangered Gazelle species (*Gazelle gazella* and *Gazelle dorcas*) and one subspecies (*Gazelle gazelle acaiae*). *Theriogenology* 2006: 66: 775–784.
11. **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.
12. **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.
13. **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.
14. **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.
15. **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.
16. **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.
17. **Paulenz H, Soderquist L, Adnoy T, Nordsroga 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.
18. **Vivanco HW, Alarcon VP**. Artificial insemination of ewes with frozen semen in the Peruvian central Andes. *Proc Western Section Am Soc Anim Sci* 1987; 38: 237–239.
19. **Matsuoka T, Imai H, Kohno H, Fukui Y**. Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen-thawed ram semen. *J Reprod Dev* 2006; 52: 675–683.
20. **Anel L, Alvarez M, Martinez-Pastor F, Garcia-Macias V, Anel E, de Paz P**. Improvement strategies in ovine artificial insemination. *Reprod Dom Anim* 2006; 41: 30–42.
21. **Bousseau S, Brillard JP, Marguant-Le GB, Guerin B, Camus A, Lechat M**. Comparison of bacteriological qualities of various egg yolk sources and the *in vitro* and *in vivo*

fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology* 1998; 50: 699–706.

22. **Bielanski A**. Disinfection procedures for controlling microorganisms in the semen and embryos of humans and farm animals. *Theriogenology* 2007; 68: 1–22.
23. **Andersen K, Aamdal J, Fougner JA**. Intrauterine and deep cervical insemination with frozen semen in sheep. *Zuchthygiene* 1973; 8: 113–118.
24. **Fukui Y**. Fukui Y (ed.), New Reproduction Technologies in Sheep. Tokyo: Tokyo Agricultural University Press; 2004: 75–80 (in Japanese).
25. **Fukui Y**. Studies in controlled sheep breeding. Ph.D. Thesis, The University of New South Wales, Sydney. 1976.
26. **Fukui Y, Roberts EM**. Further studies on no-surgical intrauterine technique for artificial insemination in the ewe. *Theriogenology* 1978; 10: 381–393.
27. **Fukui Y, Roberts EM**. Sheep intrauterine AI. *In*: Tomes GJ, Robertson DE, Lightfoot RJ (eds.), Sheep Breeding. 2nd ed, London: Butterworths & Co. Ltd.; 1979: 482–494.
28. **Killeen ID, Caffery GJ**. Uterine insemination of ewes with the aid of laparoscope. *Aust Vet J* 1982; 59: 95.
29. **Olesen I**. Effects of cervical insemination with frozen semen on fertility and litter size of Norwegian sheep. *Livest Prod Sci* 1993; 37: 169–184.