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Development of Superovulation Method in Korean Native Goats

C. Y. Choi, D. S. Son, Y. K. Kim, M. H. Han, U. G. Kweon, S. H. Choi, Y. H. Choy, S. B. Choi, Y. M. Cho, S. K. Son, G. J. Rho[1†] and S. Y. Choe[1]

Animal Genetic Resources Station, National Livestock Research Institute, R. D. A.

한국 재래산양의 과배란 처치 방법 개선

최창용 · 손동수 · 김영근 · 한만희 · 권응기 · 최순호 · 최연호 · 최성복 · 조영묵 · 손삼규 · 노규진[1†] · 최상용[1]
농촌진흥청 축산연구소 남원지소

SUMMARY

한국 재래산양 체내수정란 생산에 대한 발정동기화 및 과배란 유도방법과 회수된 수정란의 동결 융해 후 생존율을 조사하였다. 발정동기화를 위해 CIDR+FSH 및 CIDR+PMSG의 방법을 이용한 결과, 배란점 및 회수된 수정란의 수는 CIDR+FSH 처리구에서 16.3개 및 9.4개, CIDR+PMSG 처리구에서 16.4개 및 8.7개를 나타내어 두 처리구간에 유의적 차이는 인정되지 않았다. 회수된 수정란을 형태학적으로 평가한 결과 CIDR+FSH 처리구에서 Gade A, B, C 및 D는 75.8%, 15.2%, 4.5% 및 4.5%를 나타낸 반면 CIDR+PMSG 처리구에서는 52.5%, 16.4%, 16.4% 및 14.8%였으며, 이식 가능한 수정란 (Grade A, B) 수는 CIDR+FSH 처리구가 유의적($P<0.05$)으로 높게 나타났다. 회수된 수정란의 완만 동결 융해 후 생존성은 CIDR+FSH 처리구에서 73.3%, CIDR+PMSG 처리구에서 63.3%이었으며, 두 군간의 유의적 차이는 인정되지 않았다. 따라서 본 결과는 한국 재래산양 체내수정란의 생산과 회수된 수정란의 보존을 위해서 CIDR+FSH로 발정동기화 시키는 것이 효과적이었다.

(Key words : superovulation, CIDR, FSH, PMSG, Goats)

INTRODUCTION

Goats have been used as models in studies of reproductive physiology for several decades. Assisted reproductive technologies such as artificial insemination (AI) and multiple ovulation embryo transfer (MOET) have been introduced to overcome reproductive efficiencies in goats, and accelerated genetic gain. The technologies used for superovulation, collection and transfer of *in vivo* embryos are now well developed. The recent improvements of embryo production in vitro and freezing technologies could be used for constitution of flocks and will allow wider propagation of valuable genes in small ruminants population in the future. However, although MOET due to its high cost cannot replace AI as a routine reproductive technology, it can be applied to correctly choose dams to allow extra genetic gain through the production by embryo transfer of males with positive indexes

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[1] Institute of Animal Medicine, College of Veterinay Medicine, Gyeongsang National University.(경상대학교 수의과대학 동물의학연구소)

† Correspondence : E-mail : jinrho@nongae.gsnu.ac.kr

used for AI (Smith, 1986; Nicholas, 1996; Colleau et al., 1998).

Due to the seasonality of their natural breeding cycles and the need to have several donors ready at the same time for embryo collection, a superovulatory treatment is almost always preceded by estrus synchronization treatment. Because embryos may be required at any time of the year and in sufficient numbers, superovulatory treatments may have to be repeated over a number of months. For that reason a progestagen treatment precedes the gonadotrophin part of the regimen administrated to the donor. The effects of superovulation in farm animals and experimental animals were different responses owing to age of animals (Zarrow and Wilson, 1961) and season (Shea et al., 1984; Gordon et al., 1987; Choe et al., 1998). *In vivo* derived goat embryos are transferred annually within countries and internationally. The techniques used for superovulation, collection and transfer of such embryos are now well developed, but not in Korean native goats.

Several techniques and protocols for embryo freezing have been reported recently. Research has been mainly focused on bovine embryos (Edwards et al., 1997; Niemann, 1991), wherease the cryopreservation of embryos from goats (Bilton and Moore, 1976; Chemineau et al., 1986; Baril et al., 1989; Li et al., 1990) is less abundant. Advancements and factors affecting the efficiency of cryopreservation of goat embryos have been recently reported (Massip, 2001; Dobrinsky, 2002). Goat embryos are able to survive following vitrification and slow freezing procedure, and this method may provide an economical alternative to the current freezing methods that require gradual dehydration of embryonic cells (Baril et al., 2001).

The aim of the present study was to develop the estrus induction by assessments of number and quality of embryos, and viability of embryos following freezing and thawing in Korea native goats.

MATERIALS AND METHODS

1. Animals

Twenty healthy mature goats, over 2 years of age and weighing 25 to 35 kg in Namwon Branch, National Livestock Research Institute were used in these experiments. Breeding was separated to grazing season (April-October) and non-grazing season. Each buck was fed 0.4 kg of concentrated feed. Dried grass, salt lick and water supplied *ad libitum*.

2. Superovulation

Superovulation of goats was performed by the combinations of insertion of a controlled intravaginal drug releaser device (CIDR® sheep and goat, 300 mg progesterone, Pharmacia and Upjohn, New Zealand) and intramuscularly injection of FSH (Folltropin®-V, Vetrapharm, Canada) and PMSG (Folligon®, Intervet, Netherlands).

Goats inserted CIDR on day 0 of the superovulation schedule were divided into two groups. In group 1, 10 goats were injected FSH twice daily on day 12, 13 and 14 after CIDR insertion. Decreasing doses of FSH were given, as 5, 5, 3, 3, 2 and 2 mg for continued treatments each 12 h intervals. In group 2, 10 goats were injected 1,000 IU PMSG single injection on day 12 after CIDR insertion. In both of groups 1 and 2, CIDR was removed in the morning of day 14 of the treatment. At 24 h of CIDR removal, animals were mated and followed by injected 100 ug GnRH (Fertagyl®, Intervet, Netherlands). Flow chart for the procedures is presented in Figure 1.

3. Recovery of Embryos

Embryos at 7 day after mating were collected surgically under general anesthesia with the combinations of 0.05 mg/kg of xylazine HCl (Rompun®, Korea) and 2 mg/kg of ketamin HCl (Ketamine 50®, Korea) intravaneously. Both uterine horns were

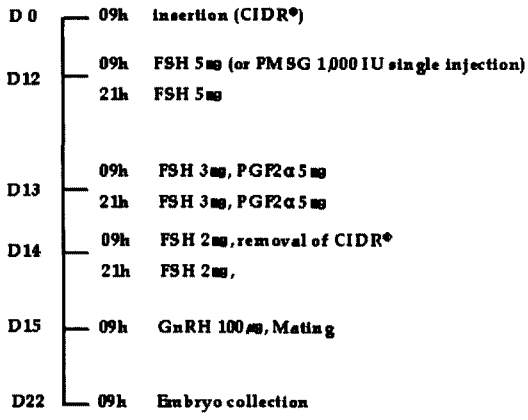

Fig. 1. Flow charts of superovulation, mating and embryo collection in Korean Native Goats.

flushed with pre-warmed phosphate buttered saline (PBS) supplemented with 5% FBS. The recovered embryos were classified into 4 grades (Grade A, B, C and D) by categorization of a previous report (Gary and Raymond, 1983).

4. Cryopreservation of Embryos Produced *in vivo*

Embryos were frozen either by vitrification or by slow freezing with different cryoprotentants which are mostly used for embryo freezing. In brief, embryos recovered from uterine horns were divided to freezing groups. In group 1, embryos were frozen in a vitrification solution (VS) which composed of Dulbecco's phosphate buffered saline (D-PBS) supplemented with 10% fetal bovine serum (FBS), 40% (v/v) ethylene glycol (EG), 18% ficoll (w/v) and 0.3 M sucrose that described by Kasai et al. (1990). Embryos exposed to VS for 1 min at room temperature (20~24℃) were loaded into a 0.25 ml plastic straws (IMV®, France) within 1 min. The straws were than placed 1 to 2 cm above the surface of the liquid nitrogen (LN_2) for 10 sec and plunged and stored into LN_2 tank. Group 2 embryos were frozen in slow freezing solution (EG) which composed of D-PBS supplemented with 10% FBS, 1.8 M EG, 5.0% (w/v) polyvinylpyrrolidone (PVP) and 0.05 M galactose. Embryos exposed to EG for 30 min at room temperature were loaded into a 0.25 ml plastic straw. The straws cooled at −2℃/min to −7℃, and held for 10 m using a cell freezer. After seeding, the straws were further cooled at −0.3℃/min to −35℃ and then plunged into LN_2 tank.

Embryo thawing was performed by shaking the straws in 37℃ water after exposure for 10 second in air. The contents of straw containing embryos were expelled into a 4-well dishes containing 1 ml of 0.5 M sucrose (vitrification) or 0.5 M galactose (slow freezing) in D-PBS supplement with 20% FBS. After 5 min, the embryos were transferred to 1 ml D-PBS for 5 min to rehydrate. Finally, the embryos were washed in mSOF medium supplemented with 0.3% BSA. The embryos were cultured for 48 h at 39℃ in a humidified atmosphere of 5% CO_2 in air to assess the viability of the embryos. All processes of dilution were conducted at room temperature.

5. Statistical Analysis

Differences were analyzed among treatments using the General Linear Model (GLM) procedure in the Statistical Analysis System (SAS). A probability of $P<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

1. Effects of Superovulation Treatments on Ovarian Response and Embryo Recovery

Ovarian response and embryo recovery in goats that were superovulated and followed by AI between two different treatments were compared as presented in Table 1. Goats on day 7 after AI were evaluated their ovarian response and recovered the embryos by flushing uterine horns with PBS. In CIDR+FSH, ovulation point and the number of recovered and fertilized embryos were 16.3, 9.4 and 6.7, and 16.4, 8.7 and 6.3 in CIDR+PMSG, res-

pectively. It did not differ between two superovulation treatments. The data reported here on ovulation points occurred in ovaries after combined treatments with CIDR is approximately 2 times higher (~16) than that reported by Majumdar et al. (1997) and Yoon et al (1997) who used only FSH (~7) and used combined treatments of norgestomet ear plant with FSH (8~10), respectively.

2. Effects of Superovulation Treatments on Embryo Quality

The present study has confirmed that combined treatments of CIDR with multiple FSH for superovulation of goats results in significantly ($P<0.05$) higher percentages of obtaining excellent grade embryos than with single PMSG, although it did not differ between two superovulation treatments on ovarian responses and number of oocytes collected. Embryos on day 7 recovered by different superovulation treatments were classified into 4 grades (Grade A, B, C and D) by morphological characteristics, as shown in Table 2. The percentages of recovered embryos classified with grade A (excellent), grade B (good), grade C (fair) and grade D (poor) were 75.8%, 15.2%, 4.5% and 4.5% in CIDR+FSH treatment and 52.5%, 16.4%, 16.4% and 14.8% in CIDR+PMSG treatment, respectively. The rates of excellent grade of recovered embryos were significantly ($P<0.05$) higher in CIDR+FSH treatment (75.8%) than that in CIDR+PMSG treatment (52.5%). Of 127 calculated embryos, 82 (64.6 %) were categorized in grade A. It has been reported that observation of embryos quality before transfer the embryos into a surrogate plays an important role on the success of pregnancy. Following the transfer of embryos in excellent, good, fair and poor categories resulted in 45%, 44%, 27% and 20% pregnancy rates (Gary and Raymond, 1983). Therefore, this experiment indicates that CIDR+FSH treatment for superovulation and high quality of *in vivo* embryo production is efficient method in Korean native goats.

3. Effects of Superovulation Treatments on Embryo Survivability after Freezing

Table 2. Effects of superovulation treatments on embryo quality

Embryos	No. of embryos (%)*		
	CIDR+FSH	CIDR+PMSG	Total
Grade A	50 (75.8)[a]	32 (52.5)[b]	82 (64.6)
Grade B	10 (15.2)[a]	10 (16.4)[b]	20 (15.7)
Grade C	3 (4.5)[a]	10 (16.4)[b]	13 (10.2)
Grade D	3 (4.5)[a]	9 (14.8)[b]	12 (9.4)

* CIDR+FSH, CIDR in for 14 days and FSH twice daily on day 12, 13 and 14 and AI on day 15.
CIDR+PMSG, CIDR in for 14 days and PMSG single on day 12 and AI on day 15.
[a,b] The value with different superscripts within the same row were significantly different ($P<0.05$).

Table 1. Effect of ovarian response and embryo recovery following different superovulation treatments (Mean±SD)

Treatments*	Number of					Recovery rate (%)
	Ovulation points	Unovulated follicles	Recovered embryos	Fertilized embryos	Unrecovered embryos	
CIDR+FSH	16.3±4.5	8.6±2.5	9.4±7.6	6.7±8.9	9.7±7.9	57.2±36.5
CIDR+PMSG	16.4±2.8	6.9±1.1	8.7±1.8	6.3±4.4	10.1±6.1	53.8±11.5

* CIDR+FSH: CIDR in for 14 days and FSH twice daily on day 12, 13 and 14 and AI on day 15.
CIDR+PMSG: CIDR in for 14 days and PMSG single injection on day 12 and AI on day 15.

Table 3. Effects of freezing methods on embryo survivability

Freezing method*	Superovulation**	No of embryos		Survival rate (%)
		Frozen	Hatched	
Slow freezing	CIDR+FSH	30	22	73.3[a]
	CIDR+PMSG	30	19	63.3[a]
Vitrification	CIDR+FSH	30	10	33.3[b]

* Slow freezing : -2℃/min to -7℃ → seeding → -0.3℃/min, to -35℃ → plunged to LN_2.
Vitrification : placed 1 to 2 cm above the surface of LN_2 for 10 sec → plunged to LN_2.

** CIDR+FSH, CIDR in for 14 days and FSH twice daily on day 12, 13 and 14 and AI on day 15.
CIDR+PMSG, CIDR in for 14 days and PMSG single on day 12 and AI on day 15.

[a,b] The value with different superscripts within the same column were significantly different ($P<0.05$).

Day 7 embryos in grade A and B which were collected from goats superovulated by two different methods were frozen either by vitrification or by slow freezing and evaluated the survivability after thawing, by assessment of hatching of the embryos. Table 3 presents the results comparing the survival rates following cryopreservation. The survival rate of embryos which were frozen by slow cooling ramp rates in CIDR+FSH treatment was slightly higher than that in CIDR+PMSG treatment (73.3% vs. 63.3%, respectively), but it did not differ. However, survival rate of embryos which were frozen by rapid cooling ramp rate was dramatically reduced to 33.3%. This rate was significantly ($P<0.05$) lower that that of slow cooling ramp rate.

CONCLUSION

In conclusion, combined treatments of CIDR+FSH multiple injection is suitable on superovulation for collecting a large number of high grade embryos, in which slow freezing can be used for the storage. The further study, in particular based on the studies of gene expression and regulation related to retardation on the development of embryos, will provide greatly enhancement of the goat IVF fields.

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