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The Evaluation of Factors Affected on Juvenile *in vitro* Embryo Transfer in Xinjiang Fine Wool Lamb

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Abstract: This research was conducted to determine the effect of FSH made in Canada and Ningbo and the ages and weight of lamb on juvenile lamb superovulation of Xinjiang Fine Wool Sheep and to compare oocyte mature time from juvenile lamb superovulation and adult ewe ovary and to study the effect of oocyte mature time and oocyte level on the quality of IVF. The results showed that the optimal method of lamb's superovulation was to inject 30 mg FSH made in Canada for four times with the interval of 12 h and the best maturation time of oocyte *in vitro* for cleavage was 30 h. The development of oocyte in different stages was not affected on cleavage. This study can provide important experimental data for juvenile *in vitro* embryo technology.

Key words: Xinjiang Fine Wool lamb, follicular development, oocyte, maturation *in vitro*, IVF

INTRODUCTION

Xinjiang Fine Wool sheep is the first Fine Wool sheep breed in China. The wool produced by them is fine, flexible and soft and is of high demand by the textile industry and has become the pillar industry of local herdsman. However, Xinjiang fine-wool sheep breeding performance is low. Therefore, to increase the number of Xinjiang Fine Wool sheep is very important for producing more wool.

Juvenile *In Vitro* Embryo Technology (JIVET) is a biological high technology involving the collection of immature eggs from young animals, their *in vitro* maturation and fertilization and the transfer of the resultant embryos into recipient females. This technology can achieve rapid generation turnover for sheep (O'Brien *et al*., 1997; Ledda *et al*., 1999; Kochhar *et al*., 2002). It has been confirmed that this technology can be applied efficiently in China.

However, in production practice, the hormone sources can influence the effect of the superovulation and the ages and weight of sheep responding to the hormone exist in a large degree of difference. Therefore, studying the hormone and ages and weight of sheep affecting on oocytes obtaining, oocytes maturation *in vitro* and fertilization *in vitro* can provide the experimental basis for improving sheep early breeding.

In this study, researchers evaluated some main factors affecting on juvenile *in vitro* embryo transfer in Xinjiang Fine Wool lamb. This study can provide important experimental data for Juvenile *in Vitro* Embryo Technology (JIVET).

MATERIALS AND METHODS

Animals: Lambs were generated from adult Xinjiang Fine Wool ewes after natural mating or embryo transfer. The lambs were 35-75 days old and the body weights were between 9-20 kg. Surgery on animals used in this study followed the regulations of animal welfare provided by the institute.

Chemicals: Follicle-Stimulating Hormone (FSH, made in Ningbo, batch number 11004462; made in Canada, batch number: L022-D083), PMSG (Ningbo, batch number 080416) and Fetal bovine serum (Hycolon, batch number SH30070.03). The other chemicals were obtained from Sigma Chemical Company (St. Louis, MO). Heat-inactivated Estrous Sheep Serum (ESS) was prepared as described by Gou.

Hormone treatment protocol and oocytes collection: The treatment protocol was performed by the following methods of Kelly *et al*. (2005) and Gou. Oocytes were

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collected approximately 12-14 h after the last time FSH treatment. Ovaries were exposed by mid-ventral laparotomy that was conduced under general anesthesia induced (IM) by 0.1 mL anesthetic mixture (Jing-Song-Ling which main component was hydrochloride, Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences). Cumulus-Oocyte Complexes (COCs) were aspirated from follicles between 2-8 mm in size using an 18 gauge needle and a 10 mL syringe. The syringe contained approximately 2-3 mL Ovum Pick-Up (OPU) liquid (Hepes buffered TCM199 containing 5 mmol L^{-1} $NaHCO_3$, 10 mmol L^{-1} HEPES, 10 mmol L^{-1} HEPES-Na, 0.01 g L^{-1} heparin sodium, 1% fetal bovine serum, 0.065 g L^{-1} Benzylpenicillin sodium and 0.05 g L^{-1} Strepolin sodium), COCs from each lamb were then transferred to 50 mL centrifuge tube and precipitated at 37°C for 10-20 min. These COCs were then immediately examined under the microscope after they were brought into the laboratory and then divided into class A-C oocytes. Lambs were awakened by 0.1 mL antagonist of adrenergic receptor-γ (Su-Xing-Ling, Institute of Military Veterinary).

Oocyte maturation *in vitro*: The oocytes were aspirated using pasteur pipet under the microscope and divided into three levels, level A-C. These oocytes were washed two times with OPU liquid and then were washed three times with maturation culture medium (Hepes buffered TCM199 containing 10% fetal bovine serum, 1 mg mL^{-1} FSH, 1 μL mL^{-1} LH, 1 μL mL^{-1} Estradiol, 1 μL mL^{-1} sodium pyruvate, 10 μL mL^{-1} penicillin-streptomycin). After washed, the oocytes were transferred to 75 μL maturation culture medium droplets and then the maturation culture medium droplets were covered with paraffin oil (Each droplet were contained about 20 oocytes). The oocytes were cultured at 38.5°C under 5% CO_2 in air for 24, 26 and 30 h.

***In vitro* fertilization procedures:** After maturation, the oocytes were treated with 0.5% hyaluronidase and stripped of excess cumulus cells by gentle pipetting, washed three times with HEPES-buffered Synthetic Oviduct Fluid (SOF) medium and then were placed in culture wells, each of which contained 400 μL of IVF medium (SOF contained 2% ESS) converted with paraffin oil.

The frozen semen was thawed using a dry sterile glass tube which was preheated at 37°C in a water bath pot with rapid shaking. After thawed, the frozen semen were put into the bottom of another glass tube which contained HEPES-buffered Synthetic Oviduct Fluid (SOF) medium and then cultured in constant temperature incubator at 37°C. After cultured for 30 min, the upper semen were transferred to 1.5 mL centrifuge tube and diluted with $NaHCO_3$-buffered SOF IVF medium containing 2% (v/v) ESS. The concentration of the sperm was determined and about 5×10^6 sperm mL^{-1} were placed in each IVF well. The oocytes and sperm were incubated together for 22-24 h at 38.5°C under 5% CO_2 condition in the constant temperature incubator.

Early *in vitro* embryo culture: Groups of 50 zygotes were washed three times in SOF culture medium and transferred into the four well culture plates, each well were contained the 500 μL embryo culture medium (SOF culture medium containing 8 mg mL^{-1} fatty acid-free BSA and amino acids). The culture plates were cultured in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C.

Statistics analysis: All data were analyzed by the Statistical Analysis System Software SPSS 10.0 one-way ANOVA which was to analyze the difference.

RESULTS AND DISCUSSION

The effect of FSH on superovulation: Three kinds of induction methods were used. FSH made in Canada Declining Method (group I), each lamb was injected FSH made in Canada at approximate 12 h intervals, the total numbers of injections were four and the dosis of the FSH was 40 IU in the 1st day and 20 IU in the 2nd day. FSH made in Canada Equivalent Method (group II), each lamb was injected 30 IU every time at approximate 12 h intervals and then the oocytes were taken from the ovary in the 3rd day. FSH made in Ningbo Equivalent Method (group III), each lamb was injected 20 IU FSH made in Ningbo every time at approximately 12 h intervals and the total numbers of injections were six. The results were showed that the average number of follicles in group I was extremely lower than that in group II and III ($p<0.01$) (Table 1) while the availability rate of recycling follicles among three groups was not significantly different ($p>0.05$) (Table 1).

The effect of different ages of lamb on superovulation: In this experiment, forty five Xinjiang Fine Wool Lambs were divided into four groups according to their ages (Table 2). Equivalent Method was used to give each lamb FSH made in Ningbo. The average number of oocyte obtained from different ages of lambs was showed in Table 2. From Table 2, it showed that the average number of follicles was significantly different between the ages of lamb from 35-45 days and from 46-55 days ($p<0.05$). The rate of available oocytes was the highest when the ages of lamb were from 46-45 days.

Table 1: Superovulaiton effect of different FSH

Groups	Number of donor lambs	Number of follicle	Average number of follicles	Nunber of recovery oocytes	Recovery rate (%)	Number of available oocytes	Available rate (%)
I	3	117	39.00±27.06[a]	75	64.10	67	89.33
II	4	404	101.00±38.32[b]	306	75.74	276	90.20
III	45	4748	105.51±55.87[b]	3149	66.32	2591	82.28

Significant difference marked with different small letters ($p<0.05$)

Table 2: The effect of day after birth on oocyte gain and availability

Age (days)	Number of lamb	Average number of follicles	Number of recovery oocytes	Number of available oocytes	Rate of available oocytes (%)
35-45	15	124.73±57.70[a]	1083	921	85.04[a]
46-55	11	125.12±67.17[a]	923	829	89.81[B]
56-65	13	83.85±59.17[b]	785	637	81.14[a]
66-75	6	88.34±40.95[b]	273	204	74.72[c]

Values with different supercase letters within same column are extremely significant difference ($p<0.01$). Values with different supercase letters within same column are extremely significant marked with the superscript within same column are significant difference marked ($p<0.05$)

Table 3: Oocytes maturation time of lambs and adult sheep *in vitro*

Cell source	Culture time (h)	Number of oocytes	Number of cleavage	Cleavage rate (%)
Lamb	24	272	131	48.16[a]
	26	161	91	56.52[ab]
	28	428	299	69.86[ab]
	30	348	262	75.29[bc]
Ovary adult eve	24	375	288	76.80[bc]

Significant difference marked with different small letters ($p<0.05$)

Table 4: The effect of oocytes level on cleavage rate and blastula rate

Levels	Number of oocytes	Rate of first polarbody (%)	Cleavage rate (%)	Rate of blastula (%)
A	192	81.25 (156)	94.87 (148/156)	54.73 (81/148)[a]
B	145	80.69 (117)	89.74 (105/117)	50.48 (53/105)[a]
C	98	85.71 (84)	85.71 (72/84)	40.28 (29/72)[a]

Values with same superscript letters are not significantly different ($p>0.05$)

Comparison of the oocytes maturation time of lambs and adult sheep *in vitro*: When maturation time was 24 h *in vitro*, the rates of oocyte cleavage of adult sheep and lamb were 76.80 and 48.16%, respectively. It was significantly different between them (Table 3) ($p<0.05$). With the cultured time prolonged, the cleavage rate of the lamb was increased. When maturation time was 30 h, the cleavage rate reached to 75.29% which was closed to the cleavage rate of the adult ewe. And the cleavage rate of oocytes was significantly different from the two different maturation time (24 and 30 h) ($p<0.05$).

The effect of oocytes level on cleavage rate and blastula rate: The effect of oocyte grade on cleavage rate was showed in Table 4. When the oocytes was cultured for 20 h *in vitro*, the rate of the appearance of the first polar body in different grades of oocytes was not significantly different ($p>0.05$). The oocyte cleavage rate was decreased with the changes of the oocyte grade but there was no significant difference in the third grade ($p>0.05$). However, the oocyte cleavage rate and blastocyst rate in oocyte grade C was the lowest.

The effect of FSH on superovulation: It has been confirmed that using hormone to treat lambs can improve the oocyte developmental competence (Ledda *et al*., 1999; Revel *et al*., 1995). The FSH is the main factor affecting superovulation. FSH made in different manufacturers may affect the sensitivity of the lamb on FSH responses. The average number of oocyte was 70.8-94.2 by using FSH made in Canda to treat Merino lambs (Kelly *et al*., 2005). While the average number of oocytes was only 20.1 by using FSH made in Italy (Ledda *et al*., 1999). The results showed that the average number of oocytes was about 101.00 by using FSH made in Canda to treat Xinjiang Fine Wool lamb. This result might be because the sheep species were different. The effect of FSH made in Canda and Ningbo on superovulation of Xinjiang Fine Wool lamb by the same induction method was not significantly different ($p>0.05$). It indicated that Xinjiang Fine Wool lamb has the same sensitivity to the two FSH. However, the rates of recovery and availability by using FSH made in Canada were higher than that by using FSH made in Ningbo. So, the FSH made in Canada was recommended to Xinjiang Fine Wool lamb for superovulation.

The effect of the age of lamb on superovulation: The degree of ovarian response in different developmental stages of lamb was different on magnitude after superovulation treatment. The number of available oocytes was negatively related to the age of lamb (Kelly *et al*., 2005; Morton *et al*., 2005). At present, it is thought that 1 month old lamb ovary is the most sensitive, 2-3 months old is weaker while 5-7 months old is weakest (Shirazi *et al*., 2005). The results showed that the age of lamb was 46-55 days was the most sensitive to FSH from which the greatest number of follicular development and the rate of available oocytes were obtained. So, in order to obtain the ideal effect of superovulation, the age of Xinjiang Fine Wool lamb was about 55 days.

Maturation time of oocyte *in vitro*: In process of oocyte *in vitro*, it can be appeared that the first polar body is released however, the nucleus is not mature. Extending the mature time will avoid this phenomenon. The results indicated that when the oocyte mature time of the lamb was 30 h *in vitro*, the cleavage rate of lambs *in vitro* was the highest (Table 3). This result was not consistent with the report by Gou who used 24 h as the mature time. Therefore, determining the mature time *in vitro* should be according to the quality of oocytes collected after specific individual's superovulation. In the production practice, the best oocyte mature time of Xinjiang Fine Wool lamb *in vitro* was 30 h.

CONCLUSION

FSH made in Canada resulted in a better quality and higher cleavage rate of oocyte in Xinjiang Fine Wool lamb. The best mature time of oocyte *in vitro* was 30 h for cleavage. The development of oocyte in different stages was not affecting on cleavage.

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