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Effects of recipient oocyte source, number of transferred embryos and season on somatic cell nuclear transfer efficiency in sheep

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Abstract
To improve the efficiency of somatic cell nuclear transfer (SCNT) in sheep, we investigated the effects of recipient oocyte source, number of transferred embryos and season on the pregnancy and live lamb rates for sheep somatic cell nuclear transfer embryos. Follicle-stimulating hormone (FSH)-stimulated ovaries produced significantly more oocytes both in total and of suitable quality for maturation culture than those without FSH treatment (from slaughterhouse). However, their in vitro maturation rates were similar. Embryos were reconstructed using adult fibroblast cells into enucleated MII oocytes. The pregnancy and term rates were significantly higher in the FSH-stimulated group than in the slaughterhouse one. Oocytes from FSH-stimulated ovaries were enucleated as recipient cytoplasm for nuclear transfer in the following experiments. The transfer of 7–9 and 11–13 embryos produced significantly higher pregnancy rates than that of six embryos. However, the former groups exhibited similar live lamb rates. FSH-stimulated ovaries produced significantly more oocytes in November and December (winter) than in May to July (summer), but the associated maturation rate did not increase. Pregnancy and term rates were significantly higher when transfer occurred in winter than in summer. In conclusion, FSH treatment produced significant benefit regarding the number and quality of collected oocytes and also for the pregnancy and live lamb rates for reconstructed embryos. However, the transfer of an appropriate number of embryos (7–13) and at an appropriate season (winter) increased pregnancy and term rates.

KEYWORDS
cloning, embryos, oocytes, season, sheep

1 | INTRODUCTION

Although 20 years have passed since the birth of Dolly the sheep, the efficiency of somatic cell nuclear transfer (SCNT) is still very low, usually 1%–3%. Inappropriate or incomplete genomic reprogramming of donor nuclei after SCNT is considered to be the main reason for this low efficiency (Keefer, 2015; Long, Westhusin, & Golding, 2014; Niemann, 2016). However, apart from intrinsic factors, such as incomplete reprogramming in SCNT embryos, the reproductive character of particular animal species, such as seasonal oestrus, may also affect recipient pregnancy outcomes and cloning efficiency. By selecting appropriate procedures, high pregnancy and calving rates

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were obtained in bovine cloning, so low overall efficiency may not be caused merely by the intrinsic features of nuclear transfer embryos (Cortez, Vajta, Valderrama, Portocarrero, & Quintana, 2018).

Recipient oocytes play an important role in donor nuclear reprogramming and early embryo development. The effect of the source of goat oocyte cytoplasts on nuclear transfer efficiency has been studied, including in oocytes from FSH-pre-treated compared with untreated females (Reggio et al., 2001), and oocytes with different genetic backgrounds (Chen et al., 2007; Liu et al., 2012). In bovine studies, the rate of blastocyst development from reconstructed oocytes was significantly influenced by the maternal lineage of oocyte donors (Bruggerhoff et al., 2002; Yang et al., 2008). The number of transferred embryos is also an important factor for nuclear transfer efficiency, as shown in research on pig (Rim et al., 2013) and goat (Liu et al., 2011). However, owing to significant species differences, it is important to find the optimal number of transferred embryos per recipient for different species. The season has also been reported to affect the efficiency of porcine nuclear transfer (Huang et al., 2013; Koo, Kang, Kwon, Park, & Lee, 2010). However, the effects of the above factors have been investigated mainly in pig, goat and bovine studies, but not in sheep, another important livestock species. Sheep are seasonal breeders and short-day animals, so season also affects their reproductive performance. In this study, we investigated the effects of oocyte source, number of embryos transferred and season (winter vs. summer) on sheep nuclear transfer efficiency to identify optimal conditions.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experiments involving animals were conducted under a protocol approved by the Animal Care and Use Committee of Tianjin Institute of Animal Science and Veterinary Medicine.

2.2 | Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

2.3 | Donor cell preparation for SCNT

An ear skin biopsy was obtained from a 1-year-old male White Dorper sheep using an aseptic procedure. The sample was cut into small blocks, which were then cultured for 6–8 days in DMEM with 10% FCS at 38.5°C in a humidified atmosphere of 5% CO_2. Then, the explants were removed. Growing cells that had reached 90% confluence were passaged and washed with Dulbecco's modified Eagle's medium (DMEM; Gibco) plus 10% foetal calf serum (FCS; Gibco). Some of the cells were used immediately for nuclear transfer, while the remainder were cultured for later use. Cells were used for nuclear transfer at 3–8 passages. The cell cycle stages at passages 5 were analysed using a Flow Cytometer (FACS Calibur, Becton-Dickinson) by PI staining. Cells were collected and adjusted to final concentration of 5×10^5. The percentages of the cells at G0/G1, S and G2/M phases of the cell cycle were determined using a multicycle programme.

2.4 | Oocyte collection and in vitro maturation

Crossbred sheep were pre-treated with a progesterone intravaginal device (300 mg progesterone per device; Pfizer Australia Pty Ltd) for 18 days. FSH (Ningbo Hormone Products Co. Ltd.) was administered twice a day at 200–220 IU in total during days 16–18 of device insertion. The device was removed after the last FSH injection. Twelve to fifteen hours later, ovaries were exposed by mid-ventral laparotomy. Oocytes were collected from stimulated ovaries by aspiration from 2- to 8-mm-diameter follicles using an 18-gauge needle. Cumulus–oocyte complexes (COCs) with at least one layer of cumulus cells were placed in TCM-199 (Gibco) collecting medium supplemented with 5% (v/v) FCS, (Gibco), 30 μg/ml heparin and 4.766 mg/ml Hepes. After washing three times, 20–40 COCs were allocated randomly to each well of four-well plates (Nunc) containing 1 ml of TCM-199 supplemented with 10% (v/v) FCS, 10 μg/ml FSH, 20 μg/ml LH (Ningbo Hormone Products Co. Ltd.) and 1 μg/ml oestradiol for culture at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 22–24 hr. COCs were also aspirated from ovaries collected from a local slaughthouse and cultured in vitro as described above for COCs from FSH-stimulated origin.

2.5 | Nuclear transfer

The SCNT procedure was carried out as described previously (Liu & Liu, 2018). Oocytes were denuded by aspirating and expelling repeatedly in 0.5% hyaluronidase for 5 min. Those with first polar bodies and normal morphology were pre-treated for enucleation in TCM-199 medium supplemented with 5 μg/ml Hoechst 33,342 and 5 μg/ml cytochalasin B for 10–15 min. Enucleation was performed by removing the first polar body and the small portion of ooplasm that contained the metaphase II spindle with an aspiration pipette mounted on micromanipulators (MMN-1, Narishige) under an inverted fluorescence microscope (TE 300, Nikon); removal was confirmed by exposure to UV light. A single donor cell was inserted inside the perivitelline space of each enucleated oocyte. The oocyte–fibroblast couplets were washed twice in fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$), moved into the fusion chamber and aligned manually. Cell fusion was induced by applying two direct current pulses of 2.0 kV/cm for 25 μs each with a 1-s interval using an ECM2001 Electrocell Manipulator (BTX Inc.). The couplets were evaluated for fusion more than 30 min after the pulses, and the fused couplets were further activated by incubation in TCM-199 medium supplemented with 10 μg/ml cycloheximide and 5 μg/ml cytochalasin B for 5 hr, followed by culture in TCM199 + 10% FCS for 13–15 hr post-fusion before transfer.

2.6 | Embryo transfer

Crossbred sheep were used as embryo recipients and synchronized by pre-treatment with a progesterone intravaginal device (the same type as used for oocyte donors). The device was removed on the

same day as in the donor sheep. The recipients were observed on day 2 after oestrus. Ovaries and oviduct were exposed by mid-ventral laparotomy. Embryos were transferred into the oviductal lumen on the side containing the corpus luteum via the fimbria using a catheter. Six to thirteen embryos were transferred into each recipient. At this timepoint, all of the embryos were at the one-cell stage. To reduce the adverse effects on embryos of in vitro culture, we transferred embryos at the one-cell stage. Pregnancy was checked 45 days after transfer and foetal development was monitored with ultrasound.

2.7 | Statistical analysis

The data were pooled from at least three replications. Data were analysed using the chi-squared test with SPSS 17.0 software, and $p < .05$ was considered significant. Data were pooled from at least three replications.

3 | RESULT

3.1 | The cell cycle stages analysing for donor cells

Flow cytometry analysis revealed that 75.35% of donor cells at passages 5 were at G0/G1 phase, 12.4% at S phase and 12.61% at G2/M phase (Figure 1).

3.2 | Effect of recipient oocyte source on in vivo development of reconstructed embryos

A total of 267 oocytes were collected from 17 FSH-stimulated donor ewes (34 ovaries). An average of 7.8 oocytes were collected per stimulated ovary, and 217 oocytes of suitable quality (6.4 oocytes/ovary) for maturation culture. A total of 243 oocytes were recovered from 152 slaughterhouse ovaries (1.6 oocytes/ovary, not treated with FSH) and 178 oocytes of suitable quality (1.2 oocytes/ovary) for maturation culture. FSH-stimulated ovaries produced significantly more oocytes (total and of suitable quality) than slaughterhouse ovaries ($p < .05$). In addition, the pregnancy rate (6/15, 40% vs. 1/7, 14.3%, $p < .05$) and term rate (2/6, 33% vs. 0, $p < .05$) were significantly higher, but the pregnancy loss rate was significantly lower (4/6, 66.7% vs. 1/1, 100%, $p < .05$). However, no significant difference was found between the two treatment groups in term of the maturation rate (183/217, 84.3% vs. 127/178, 71.3%, $p > .05$), fusion rate of reconstructed embryos (131/151, 86.7% vs. 86/108, 79.6%, $p > .05$) and live lamb rate (2/131, 1.5% vs. 0/86, 0, $p > 005$) after SCNT embryo transfer (Table 1).

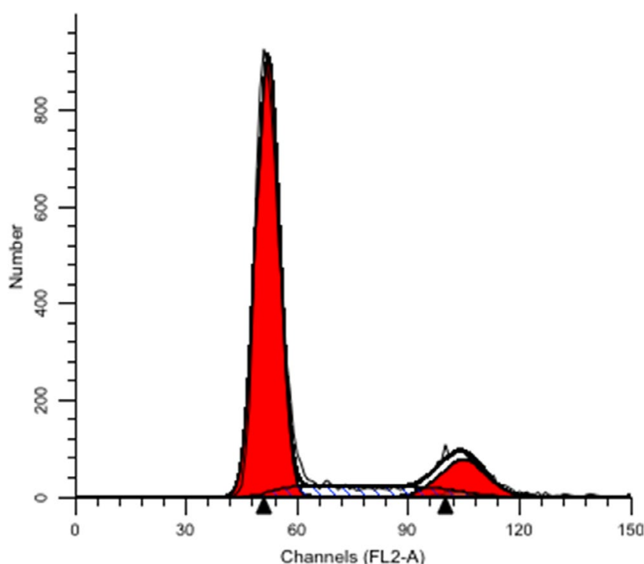

FIGURE 1 Flow cytometry analysis revealed that 75.35% of donor cells at passages 5 were at G0/G1 phase, 12.4% at S phase and 12.61% at G2/M phase

3.3 | Effect of number of transferred embryos on in vivo development of reconstructed embryos

Embryos were reconstructed with oocytes collected from FSH-stimulated ovaries. To investigate how the number of transferred reconstructed embryos affects nuclear transfer efficiency in sheep, we set up three experimental groups: those transferred with 6 embryos, 7–9 embryos and 11–13 embryos. No pregnancy was established in recipient ewes (0/7) into which 6 embryos were transferred. The pregnancy rates of those receiving 7–9 embryos and 11–13 embryos were similar ($p > .05$), but significantly higher than those with 6 embryos (10/27, 37%; 6/12, 50% vs. 0/7, 0% $p < .05$). However, the rate of pregnancy loss was significantly higher in the group with 7–9 embryos group than in the group with 11–13 embryos (8/10, 80% vs. 3/6, 50%, $p < .05$). No significant differences were found in the live lamb rates between the two groups (0%; 2/220, 0.9%; 3/139, 2.3%, respectively, $p > .05$; Table 2). The average birthweight of cloned lambs was 4.6 ± 0.9 kg.

3.4 | Effect of season on in vivo development of reconstructed embryos

Embryos were reconstructed with oocytes collected from FSH-stimulated ovaries in different seasons. A total of 543 oocytes were collected from 60 FSH-stimulated donor ewes (120 ovaries) in May to July (summer). An average of 4.5 oocytes were collected per stimulated ovary, and 453 oocytes (3.8 oocytes/ovary) were considered suitable for maturation culture. A total of 324 oocytes were recovered from 20 FSH-stimulated donor ewes (40 ovaries) in November and December (winter), and 236 oocytes (5.9 oocytes/ovary) were considered suitable for maturation culture. In May–July, FSH-stimulated ovaries produced significantly more oocytes (total and number suitable for maturation culture) than in November–December ($p < .05$). However, no significant difference was found between the seasons in terms of maturation rate (364/453, 80.4% vs. 205/236, 86.9%, $p > .05$), fusion rate of reconstructed embryos (244/310, 78.7% vs. 157/183, 85.8%, $p > .05$), pregnancy loss rate (5/6, 83.3% vs. 6/10, 60%, $p > .05$) and live lamb rate (1/244, 0.4% vs. 4/157, 2.5%, $p > .05$). The pregnancy rate (10/17, 58.8% vs. 6/29, 20.7%, $p < .05$) and term rate (4/10, 40% vs. 1/6, 16.7%) were significantly higher in

TABLE 1 Effect of recipient oocyte source on in vivo development of reconstructed embryos

Oocyte sources	No. of ovaries	No. of total oocytes (per ovary)	No. of qualified for maturation (per ovary)	No. of matured (%)	No. of reconstructed	No. of fused (%) (transferred)	No. of recipients	No. of pregnant (%)	No. of loss (%)	No. of termed (%)	No. of live lambs (% embryos)
FSH-stimulated ovaries	34	267 (7.8)[a]	217 (6.4)[a]	183 (84.3)	151	131 (86.7)	15	6 (40)[a]	4 (66.7)[a]	2 (33.3)[a]	2 (1.5)
Slaughterhouse ovaries	152	243 (1.6)[b]	178 (1.2)[b]	127 (71.3)	108	86 (78.4)	7	1 (14.3)[b]	1 (100)[b]	0[b]	0

Note: Different superscripts within the same column (e.g. a, b) represent significant differences ($p < .05$).

TABLE 2 Effect of number of transferred embryos on in vivo development of reconstructed embryos

No. of transferred embryos/recipient	No. of total embryos	No. of recipients	No. of pregnant (%)	No. of loss (%)	No. of live lambs (% embryos)
6	42	7	0[a]	0	0
7–9	220	27	10 (37)[b]	8 (80)[a]	2 (0.9)
11–13	139	12	6 (50)[b]	3 (50)[b]	3 (2.3)

Note: Different superscripts within the same column (e.g. a, b,) represent significant differences ($p < .05$).

November–December than in May–July (Table 3). Pregnancy loss occurred nearly through the whole pregnancy period (Table 4).

4 | DISCUSSION

Enucleated metaphase II oocytes are generally chosen for use as recipient cytoplasm for somatic cell nuclear transfer. In a previous study on goat, significantly more oocytes were collected from FSH-stimulated than from abattoir-derived ovaries (12.2 vs. 0.81 oocytes/ovary; Reggio et al., 2001). We obtained similar results in the present study. Reggio et al. (2001) reported that no significant difference was found in the pregnancy rate between these two groups. This is different from our results showing that oocytes from FSH-stimulated ovaries were associated with a significantly higher pregnancy rate than slaughterhouse oocytes. In a recent study on camel by Wani, Hong, and Vettical (2018), embryos reconstructed with in vitro matured oocytes collected from a slaughterhouse were associated with significantly lower cleavage (55.2 ± 7.6%) and blastocyst rates (20.5 ± 5.5%) than in vitro (71.3 ± 1.3% and 36.7 ± 7.3%) or in vivo matured (91.7 ± 8.3% and 35.4 ± 6.0%) oocytes obtained from live animals ($p < .05$) pre-stimulated with FSH. This result is similar to the finding in the present study. Wani et al. (2018) proposed the possible reason for the decreased developmental potential; namely, oocytes from slaughterhouse ovaries were usually from old, unproductive or very young camels. The ovarian follicles from such animals lack normal pre-ovulatory development, such as selection and growth. In contrast, the oocytes collected by OPU were from pre-ovulatory follicles of live animals with FSH stimulation. FSH can promote higher developmental competence of oocytes. We think that this situation prevailed in sheep in this study. The reconstructed embryos from in vitro- or in vivo- matured goat oocytes were associated with similar pregnancy rates, but more foetuses were aborted in the former group (Wan et al., 2012). There were no differences between the different types of oocyte source in terms of on the establishment of pregnancies and the delivery of offspring (Wani et al., 2018).

TABLE 3 Effect of season on in vivo development of reconstructed embryos

Season	No. of stimulated ovaries	No. of total oocytes (per ovary)	No. of qualified for maturation (per ovary)	No. of matured (%)	No. of reconstructed	No. of fused (%) (transferred)	No. of recipients	No. of pregnant (%)	No. of loss (%)	No. of termed (%)	No. of live lambs (% embryos)
May to July (summer)	120	543 (4.5)[a]	453 (3.8)[a]	364 (80.4)	310	244 (78.7)	29	6 (20.7)[a]	5* (83.3)	1 (16.7)[a]	1 (0.4)
November and December (winter)	40	324 (8.1)[b]	236 (5.9)[b]	205 (86.9)	183	157 (85.8)	17	10 (58.8)[b]	6† (60)	4 (40)[b]	4 (2.5)

Note: Different superscripts within the same column (e.g. a, b) represent significant differences ($p < .05$).
*One of five pregnancy loss recipients aborted with twins.
†Two of six pregnancy loss recipients aborted with twins.

TABLE 4 Pregnancy loss during different periods of pregnancy

Season	No. pregnant	No. loss (%) Between day 45 ~ 90	between day 91 ~ 140	Total loss (%)
May to July (summer)	6	2 (33.3)	3[a] (50)	5 (83.3)
November and December (winter)	10	2 (20)	4[b] (40)	6 (60)

[a]One of three pregnancy loss recipients aborted with twins.
[b]Two of four pregnancy loss recipients aborted with twins.

In sheep or goat studies, reconstructed embryos at the one- or two-cell stage are usually transferred into the recipient oviduct less than 24 hr after activation to decrease the detrimental effect of long-term in vitro culture to the morular or blastocyst stage. However, the optimal number of embryos per recipient remains to be determined. Liu et al. (2011) reported a higher pregnancy rate upon transferring 40 rather than 10 goat embryos (73% vs. 22.6%, $p < .05$); however, the total rates of SCNT efficiency did not differ significantly (1.1% vs. 0.9%, $p > .05$). Moreover, multiple foetuses are known to have adverse effects on postnatal development (Dwyer, Calvert, Farish, Donbavand, & Pickup, 2005; Grazul-Bilska et al., 2006). In the current study, we found that at least 7 embryos were needed to establish pregnancy in sheep, although the pregnancy and live lamb rates were equivalent between the groups receiving 7–9 and 11–13 embryos; however, there was a trend for more pregnancies and live lambs when more embryos were transferred. However, when two 1- or 2-cell embryos from in vivo fertilization were transferred into recipients in Dorper sheep, the pregnant rate was 45.6% (36/79; unpublished data), similar to this present study; however, 25.8% (41/158) of the live lamb rate was far higher than that of present 2.3% (3/139). Nonetheless, it is important to prioritize total nuclear transfer efficiency (live lambs/embryo) when determining the appropriate number of embryos to be transferred.

Sheep have a distinct breeding season. Ovine oestrous cycles generally start and end when the day length is decreasing and increasing, respectively. Our results showed that season had a significant effect on pregnancy and live lamb rates, which were significantly higher when embryos were transferred in November–December (winter) than in May–July (summer). This trend follows the natural seasonal regulation of oestrous cycling. Our results are consistent with a recent report describing Dorper sheep embryo transfer (Bergstein-Galan, Weiss, & Kozicki, 2019). In that study, the season had a significant effect on the recipient pregnancy rate, with higher pregnancy rates reported in winter (65.57% ± 25.33%) than in spring (37.11% ± 33.27%), summer (29.95% ± 28.33%) or autumn (35.03% ± 31.66%). Therefore, the survival of sheep embryos is significantly higher when implanted during the breeding season. In pig SCNT studies, there was a higher full-term pregnancy rate in spring than in summer and winter, and it was suggested that spring and fall may be suitable seasons for SCNT (Huang et al., 2013). The pregnancy rate in the winter group was the lowest among all four seasonal groups, with no offspring produced (Koo et al., 2010). However, this finding differed from those in a previous report describing naturally mated pigs, which exhibited similar reproductive ability between spring and winter (Tast, Peltoniemi, Virolainen, & Love, 2002). The authors concluded that reduced pregnancy and delivery rates in winter were caused by exposure of the embryos to a low ambient temperature (Huang et al., 2013; Koo et al., 2010). The discrepancy between our results and these early reports may be due to species difference. For example, porcine oocytes have high intracellular lipid content, which may make them sensitive to temperature (Li et al., 2006).

In our study, although a high pregnancy rate was attained in winter, total nuclear transfer efficiency was low. Pregnancy loss occurred in more than half of the recipient ewes and may have been related to intrinsic factors, such as inappropriate or incomplete genomic reprogramming of donor nuclei after SCNT. Matoba et al. (2014) identified histone H3 lysine 9 trimethylation (H3K9me3) of the donor cell genome as a major epigenetic barrier for efficient reprogramming by SCNT. Reprogramming-resistant regions that were enriched for H3K9me3 modification were identified in SCNT embryos. The expression of human H3K9me3 demethylase Kdm4d reduced the level of H3K9me3 and significantly improved the efficiency of both mouse and monkey SCNT (Liu et al., 2018; Matoba et al., 2014). Other research found that the nucleus of a sheep somatic cell can be formatted into a spermatid-like structure by the heterologous expression of the *Prm1* gene, resulting in significantly greater numbers of SCNT embryos reaching the blastocyst stage (Iuso et al., 2015).

In conclusion, FSH treatment had produced significant beneficial effects in terms of the number and quality of collected oocytes, also on pregnancy and term rates for reconstructed embryos. However, the transfer of an appropriate number of embryos (7–13) and at an appropriate season (winter) increased pregnancy and term rates.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Yixin Yuan, Ruming Liu, Haijun Liu and Xianfu Zhang performed the design of the study and nuclear transfer, and prepared the manuscript; Xiaosheng Zhang, Jinlong Zhang, Zi Zheng, Chengjun Huang and Guifang Cao treated sheep, collected oocytes and transferred nuclear transfer embryos into recipients.

DATA AVAILABILITY

No additional unpublished data are available.

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